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Original Article

Preparation, characterization and stability investigation of chitosan nanoparticles loaded with the *Echis carinatus* snake venom as a novel delivery system

Mohammadpour Dounighi[*1], N., Mehrabi[2], M., Avadi[3], M.R., Zolfagharian[1], H., Rezayat[4], M.

1. Department of Venomous animals and Human Sera, Razi Vaccine & Sera Research Institute, Karaj, Iran
2. Department of Nanotechnology, Faculty of Advanced Sciences and Technology in Medicine, Tehran University of Medical Science, Tehran, Iran
3. Research and Development Department, Hakim pharmaceutical company, Tehran, Iran
4. Department of Pharmacology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Received 07 October 2014; accepted 03 December 2014
Author for correspondence. Email: Nasser_mohammadpour@yahoo.com

ABSTRACT

The chitosan nanoparticles used widely as a drug delivery systems recently. In the present study the *Echis carinataus* snake venom loaded chitosan nanoparticles were prepared based on ionic gelation of tripolyphosphate and chitosan. The nanoparticles physicochemical characteristics, stability and biological activity of encapsulated venom were studied. The particles were spherical in shape and the tripolyphosphoric groups of TPP were linked to the ammonium groups of chitosan. Optimum particle size of chitosan and venom loaded nanoparticles were 89 and 116 nm, respectively. Optimum loading capacity and loading efficiency obtained by 500 µg/ml concentration of venom. The biological activity of venom remained intact during nanoparticle formation and showed no considerable reduction in stability analysis. Our results suggested that chitosan nanoparticles, which prepared in this work possibly, could be used as an alternative for traditional adjuvants.

Keywords: chitosan nanoparticles, tripolyphosphate, venom, ionic gelation, stability

INTRODUCTION

The chitosan nanoparticles due to biocompatibility, biodegradability, mucoadhesive, non-toxic and hydrophilic properties widely used as a drug delivery system recently (Goycoolea *et al* 2007, Amidi *et al* 2006, Zhang *et al* 2008, Mansouri *et al* 2004, Hu *et al* 2002). In the pharmaceutical research the successful delivery of protein drugs has been an ongoing topic (Sugimoto *et al* 1998, Grenha 2005, Janes & Alonso 2003, Calvo *et al* 1997). The natural polymers such as chitosan and alginate studied extensively for preparing a carrier for therapeutic protein molecules and antigens in recent years (Gan & Wang 2007, Mohammadpour dounighi *et al* 2010, Borges 2005, Xu & Du 2003). Chitosan is a polysaccharide which is obtained from relative chitin deacetylation (Mohammadpour dounighi *et al* 2010, Wilson *et al* 2010). Chitosan [α (1→4) 2-

amino 2-deoxy β-D-glucan] is the second abundant polysaccharide and a cationic polyelectrolyte present in nature (Wu *et al* 2005, Wang *et al* 2007). Chitosan, a natural polysaccharide, is an N-deacetylated derivative of chitin which can be obtained from crustaceans, insects, fungi etc (Boonsongrit *et al* 2006). Chitosan is a non-toxic, biocompatible, biodegradable, haemostatic, bacteriostatic, anticancerogen, tissue compatible polymer and has immunostimulation activity as well as good mucoadhesive properties in acidic environments (Mohammadpour dounighi *et al* 2010, Xu *& Du* 2003, Sadeghi *et al* 2008, Dutta *et al* 2004, Ma *et al* 2002). Due to amine groups of chitosan it has many applications in pharmaceutical industry. Chitosan has higher positive charge than to other natural polymers; therefore, it is used extensively in drug delivery systems (Mohammadpour dounighi *et al* 2010). It is insoluble in aqueous solution at the pH≥6, except for low molecular weight samples. Chitosan can be solved in aqueous acidic media and it is related to its amine groups that are protonation (Momin 2008). Protonation of the $–NH_2$ function on the C-2 position of the D-glucosamine repeat unit results in the solubilization of chitosan, and subsequently chitosan is converted to polyelectrolyte (Rinaudo 2006). In addition, chitosan has the ability to sustain drug release in drug delivery systems (Mohammadpour dounighi *et al* 2010, Wang *et al* 2008). Pharmaceutical nanotechnology has focused on nano-drug forms such as nanoparticles, nanocapsules, micellar systems and conjugates (Patel & Jivani 2009, Uchegbu 2006). Chitosan has many advantages, particularly for developing micro/nanoparticles (Mohammadpour dounighi *et al* 2010). The most applied method for preparing chitosan nanoparticles *are* bottom-up approaches which are a result of a self–assembling or cross linking interactions. In these nanostructures, drugs are entrapped inside nanoparticles or can be attached to the matrix (Goycoolea *et al* 2007). linear polyamine structure of chitosan , which has a lot of amine groups, can interact with negative groups of different material such as pentasodium tripolyphosphate (TPP)(Mohammadpour dounighi *et al* 2010, Wang *et al* 2007). One of the most capacity of these nanoparticles is entrapping protein molecules and an improvement of peptide absorption by several mucosal routes (nasal & ocular) (Grenha *et al* 2005, Wang *et al* 2008, Pan *et al* 2002). Snake venoms are mixtures of many different pharmacologically active proteins, such as enzymes, toxins or nerve growth factors (Al Saleh 2002). The purpose of this study is to introduce a new type of antigen carrier as an adjuvant. In the present work the chitosan nanoparticle contain *Echis carinatus* venom were prepared and its specifications such as loading capacity, loading efficiency, stability of the nanoparticles and the biological activity of the venom were evaluated.

MATERIALS AND METHODS

Chitosan (low molecular weight, Mw 23kDa) with degree of deacetylation (DD) of which was >95% purchased from Primex Co. Its molecular weight (Mw) was obtained from viscosity measurements and the Mark-Houwink relationship. Sodium tripolyphosphate (STPP) was purchased from Sigma and acetic acid from Merck Co. *Echis carinatus* snake venom was provided as a freeze-dried powder by Razi Vaccine and Serum Research Institute (Karaj, Iran). Coomassie brilliant blue G250 was purchased from Sigma Chemical Co. All other materials and solvents used in this study were of analytical grade. The lethal activity of the *Echis carinatus* venom was determined according to the method of Theakston and Reid (Theakston 1983). The lethality activity of venom was estimated by injecting varying dilutions of venom in normal saline in mice. Aliquots (0.5 ml) of venom dilutions were injected intravenously into groups of four mice (17-21 g). Deaths were recorded over 96 hr and the toxicity was estimated. The toxicity of the venom under test is expressed in terms of the LD_{50}. chitosan nanoparticles were prepared according to ionic gelation of CS with TPP anions. Chitosan was dissolved at different concentrations (2, 3, 4 mg/ml) in aqueous solution of acetic acid. The concentration of

acetic acid in aqueous solution was equal to chitosan in all the case. Sodium tripolyphosphate solution (1mg /ml) was prepared. Then drops of TPP solution were added to chitosan solution. Nanoparticles were spontaneously formed by the rapid mixing of 5.7 ml of a TPP solution into 14.3 ml of the CS solution in a test tube under magnetic stirring (~1200 rpm) at room temperature. Agitation was maintained for 60 min to allow complete stabilization of the nanosystem until opalescent suspension obtained. In this condition the maximum nanoparticles were formed. Finally nanoparticles were separated by centrifuge at 11200 rpm, 4 °C for 30 min; freeze dried and, then stored at 4-8 °C. The snake venom-loaded nanoparticles were formed by the addition of various concentrations of venom to TPP solution prior to the incorporation of chitosan solution. The effects of snake venom initial concentration (100, 200, 300, 400, 500 µg/ml) in loading efficiency and also different concentration of chitosan (2, 3, 4 mg/ml) in nanoparticles size were studied. In order to evaluate one factor, its own parameter changed, and the other factors remained constant. In addition, to above mentioned parameter the other factors such as structure, morphology, surface charge, venom biological activity and nanoparticles stability were studied. In order to study nanoparticle size and morphology Scanning Elecron Microscopy (SEM) (HITACHI, S-4160) and Zetasizer (Malvern, UK) were used, respectively. Particle size was evaluated on the basis of NMD (Number mean diameter). The nanoparticles structure were studied by FTIR (Fourier Transform Infrared). FTIR was taken with KBr pellets. The nanoparticles were separated by centrifugation (11200rpm, 30 min, 4 °C) and amount of free venom in the supernatant was determined by Bradford assay (micro & macro)(Bradford 1976). Venom encapsulation efficiency (AE) and venom loading capacity (LC) were calculated as follows (Amidi 2006, Wu *et al* 2005, Dustgania 2008, Avadi *et al* 2010).

$$AE = \frac{\text{Total venom} - \text{Free venom}}{\text{Total venom}} \times 100$$

$$LC = \frac{\text{Total venom} - \text{Free venom}}{\text{Nanoparticles weight}} \times 100$$

In vitro release of venom from nanoparticles was studied. Different tubes containing 1 mg of freeze-dried nanoparticles and 1 ml of phosphate buffer (pH=7.4) were prepared. These tubes were incubated in shaker incubator at 37 °C and 150 rpm. One sample was taken at predetermined time intervals 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h and centrifuged at 14000 rpm for 30 minutes. The protein concentration in the supernatant was measured by Bradford method. The stability of venom loaded nanoparticles was analyzed on the base of accelerated condition (25 °C ± 2 °C/60% RH ± 5% RH) for 4 months(U.S. Department of Health and Human Services Food and Drug Administration 2003). The samples were taken periodically to study venom activity, release and characteristic of nanoparticles.

RESULTS

The FTIR spectra of chitosan, chitosan-TPP nanoparticles, and venom loaded nanoparticles are shown in the figure 1. In the chitosan spectra the strong peak in the 3200–3400 cm^{-1} ranges correspond to combined peaks of O-H stretching and intermolecular hydrogen bonding. The N-H stretching from primary amines are overlapped in the same region, the absorption band of the carbonyl (C=O) stretching of the secondary amide (amide I band) is shown at 1654 cm^{-1} and peak at 1073 cm^{-1} belongs to the C-O-C stretching. The spectrum of chitosan-TPP nanoparticles is different from that of chitosan matrix. In chitosan-TPP nanoparticles the peak of 3399 cm^{-1} becomes wider. In chitosan-TPP nanoparticles, the 1654 cm^{-1} peak of –NH2 bending vibration shifts to 1540 cm^{-1} and a new sharp peak 1635 cm^{-1} appears. The spectrum of loaded chitosan nanoparticles do not show any changes as compared with The spectrum of chitosan nanoparticles. In the present study, to prepare nanoparticles the different concentrations of Chitosan (2, 3, 4mg/ml) with TPP concentration 1mg/ml were investigated. Our results showed that the nanoparticle which prepared by

cconcentration of 2mg/ml have a smaller size and better polydispersity index (Table1). The SEM image of chitosan nanoparticle and venom loaded-chitosan nanoparticles showed in the figure 2 (A, B).

Table.1. The influence of chitosan concentration on size of nanoparticles (TPP 1mg/ml).

Chitosan mg/ml	Number mean diameter (Nmd)	Polydispersity index(PDI)
2	89	0.42
3	132	0.78
4	276	0.51

The particles are spherical in shape and smooth surface. The nanoparticles were evaluated by DLS and our results (Figure 3A, B) showed the particule size of chitosan and venom loaded nanoparticles 89 and 116 nm respectively. The effect of different concentration of snake venom on encapsulation efficiency and loading capacity revealed in figure 4 (A, B). The optimum loading capacity and encapsulating efficiency obtained at 500 µg/ml concentration of venom. The release profile of venom from chitosan nanoparticles showed in figure 5. A burst effect of venom release in the initial steps of the study (about 31% up to 12 hours) was observed. Zeta potential of chitosan nanoparticles and loaded nanoparticles were investigated in the beginning and the stability analyzed after 4 months (Table 2).

Table 2. Zeta potential of chitosan nanoparticles, *Echis carinatus* snake venom loaded nanoparticles at the zero time , two and four months incubation in accelerated stability test conditions (25 °C ± 2°C , RH 60% ± 5%) (Chitosan 2mg/ml, TPP 1 mg/ml and venom 500µg/ml).

	Zeta Potential (mV)
chitosan nanoparticles	+42
Echis carinatus snake venom-loeded nanoparticles	+35
Echis carinatus snake venom loaded nanoparticles after two months	+25.8
Echis carinatus snake venom loaded nanoparticles after four months	+10.8

As it is shown in the table 2 the chitosan nanoparticles have positive charge which reduced after four months incubated at accelerated stability test condition. SEM images of the chitosan loaded nanoparticles and their morphology after two and four months are shown in the figures 6 (A, B). Our results indicated that the shape of venom loaded nanoparticles remained intact after 2 months and the partially aggregated after 4 months. The toxicity (LD_{50}) of crude *Echis carinatus* venom was 40 µg per mice**.** After stability incubation at zero and four months the encapsulated venom released and toxicity estimated in-vivo (Table 3).

Table3. The In-vivo bioactivity test results for stability studies samples of Echis carinatus snake venom loaded chitosan nanoparticles (Chitosan 2mg/ml, TPP 1mg/ml and venom 500µg/ml) (positive control: venom solution in distilled water, negative control: supernatant of chitosan nanopartiles, sample1: venom released at zero time, sample 2: venom released after four months incubation, test animals: mice 18-20 gr, injection route: IV, number of animals in each group: 4

Test samples	**Negative control**		**Positive Control**		**Sample 1**		**Sample 2**	
Time (months)	0	4	0	4	0	4	0	4
Bioactivity (LD_{50})* (µg)	0	0	40	40	40	-	-	50

* Median lethal dose

The biological activity of venom after nanoparticulation process was 40 mice LD_{50} and remained intact. The toxicity of loaded venom samples tacked after 4 month incubation was 50 mice LD_{50} and not shown considerable reduction during accelerated stability analysis.

DISCUSSION

The spectrum of chitosan nanoparticles is different from the chitosan which in the chitosan nanoparticles peak of 3399 cm^{-1} becomes wider, which indicating that hydrogen bonding is enhanced (Wu *et al* 2005) and the 1654 cm^{-1} peak of –NH2 bending vibration shifts to 1540 cm^{-1} and a new sharp peak 1635 cm^{-1} appears as it is shown in the figure 1.

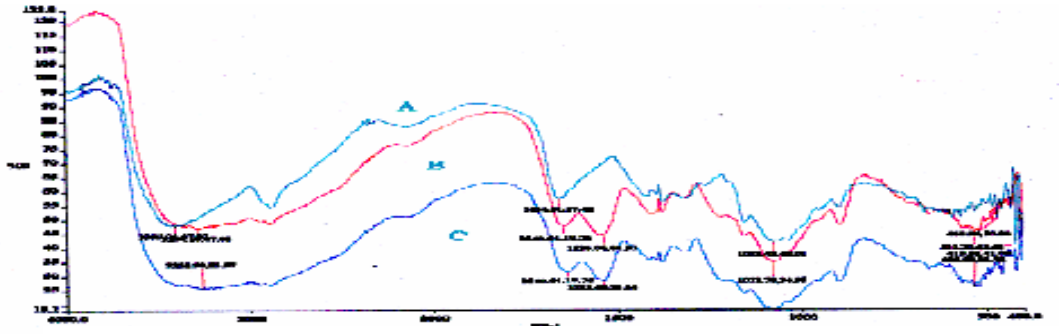

Figure 1. FTIR spectra of chitosan (A), chitosan nanoparticles (chitosan 2 and TPP 1mg/ml) (B) and *Echis carinatus* snake venom loaded nanoparticles (Chitosan 2, TPP 1mg/ml and Venom 500 µg/ml) (C).

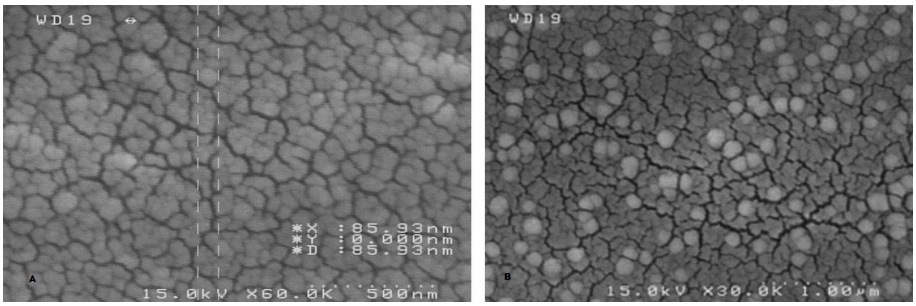

Figure 2. SEM images of Chitosan (A) and *Echis carinatus* snake venom loaded chitosan nanoparticles (B) (Chitosan 2mg/ml, TPP 1 mg/ml and venom 500µg/ml).

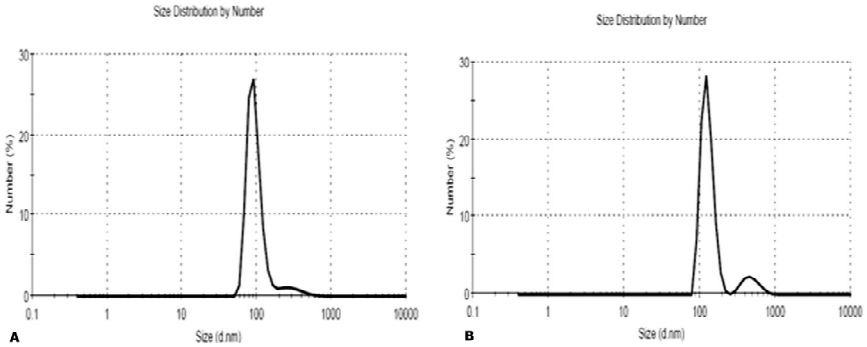

Figure3. Size distribution by number of Chitosan nanoparticles (Chitosan 2mg/ml and TPP 1 mg/ml) (A), and *Echis carinatus* snake venom loaded chitosan nanoparticles (Chitosan 2mg/ml, TPP 1 mg/ml and venom 500µg/ml) (B).

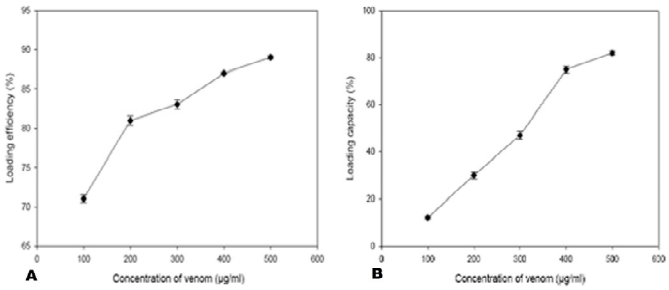

Figure 4. the influence of concentration of *Echis carinatus* snake venom on loading efficiency (A) and loading capacity (B) (Chitosan 2mg/ml and TPP 1 mg/ml).

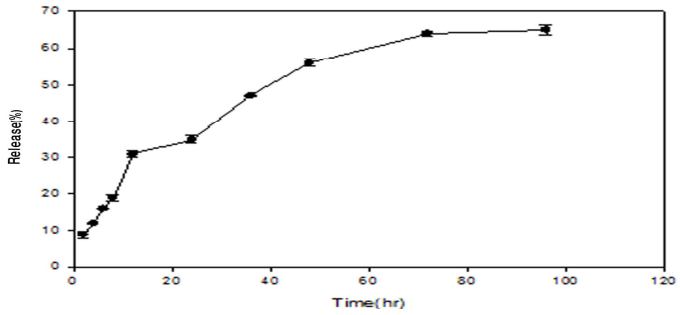

Figure 5. In vitro release profile of venom from *Echis carinatus* venom loaded chitosan nanoparticles (Stirring rate 150 rpm , pH:7.4) (Chitosan 2mg/ml, TPP 1 mg/ml and venom 500µg/ml) .

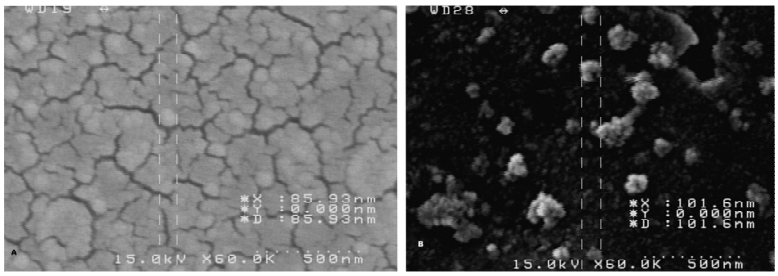

Figure 6. SEM images of Echis carinatus snake venom loaded chitosan nanoparticles (Chitosan 2mg/ml, TPP 1 mg/ml and venom 500µg/ml) incubation (A) two months and (B) four months in stability condition.

The FTIR spectrum of chitosan nanoparticles is consistent due to chitosan film modified by phosphate, and it could be attributed to linkage between phosphoric and ammonium ion. The ammonium groups of chitosan are linked with tripolyphosphoric groups of TPP in nanoparticles. Moreover, no difference was observed between loaded chitosan nanoparticles with chitosan nanoparticles. The chitosan and venom loaded nanoparticles are spherical in shape and smooth surface (Figure 2A and B), whereas the size of venom loaded chitosan nanoparticles is larger than chitosan nanoparticles, possibly, it is due to the effect of molecular weight and size of the different molecules of venom. Our results showed that the size of particles which estimated by SEM are smaller than DLS, it seems, as the samples were evaluated as a dry form in the SEM, therefore due this reason the particles observed smaller (Figure 3A and B). Venom loading efficiency and loading capacity were significantly affected by the initial venom concentration (Figure 4A and B). The loading efficiency increased from 71% to 89% and loading capacity also increased from 12% to 82% as the concentration of venom increased. Protein adsorption and encapsulation by electrostatic interactions normally exhibit a saturation kinetics which reaches a peak value at high concentrations under constant temperatures (Gan *et al* 2007). Optimum loading capacity and encapsulating efficiency of venom were obtained with concentration of 500µg/ml. It can be suggested that the positive charge of chitosan leads to electrostatic intraction between venom and polymer and results in increase encapsulation of the venom proteins (Gan *et al* 2007, Hong-liang 2009). According to the above statement and other reports a part of venom can entrapped inside the nanoparticles and other part adsorbed on the surface of nanoparticles (Gan *et al* 2007). The invitro release profile of venom nanoparticles shown in the Figure 5. In the first 31 hours of incubation about 31% of the venom was released, then followed by a very slow

venom release. Initial release of the venom is due to the venom molecules dispersing close to the nanoparticles surface, which easily diffuse in the initial incubation time. The release profile was characterized by an initial burst effect, followed by a continuous and sustained release phase. The Zeta Potential of chitosan nanoparticles and chitosan loaded nanoparticles which show a reduction of Zeta Potential from +42 to +35 mv (Table 2). Our results showed that the zeta potential of the particles slightly decreased by loading of the venom. This reduction can be due to interaction of venom with polymer and molecules of venom adsorbed on the surface of the particles (Gan & Wang 2007). The carboxyl groups on the surface of a large protein molecule may form hydrogen bond with amine groups at certain sites at the spread chitosan chain but, still maintaining a compact 3-D structure without spreading at the solution pH condition (pH 4.7) so as to keep an inner hydrophobic core. Therefore, protein molecule attachment did not sufficiently suppress the positive surface charge of chitosan molecules. During stability studies of nanoparticles, the physicochemical and biological properties of the nanoparticles (zeta potential, morphology and biological activity) were monitored. The results obtained for zeta potential of loaded nanoparticles on stability are show in table 2. Our observation indicated that decreasing trend in zeta potential which may be an attributed to change in venom 3-D structure, aggregation and morphological changes of nanoparticles. Morphology of loaded chitosan nanoparticles was evaluated at two and four months during the stability test (Figure 6A and B). It is noticed that the shape of nanoparticles has not changed after two months as compared with zero time, but significant change were observed after four month. Zeta potential of the chitosan loaded nanoparticles can greatly influence their stability in media through electrostatic repulsion between the particles. The stability of venom during encapsulation and storage of venom loaded nanoparticles is very important technical concept. Therefore, in this study at zero time and four months during stability study samples were taken from venom released (invitro) and evaluated for bioactivity invivo. The results showed that the biological activity of venom after loading process not changed (40 mice LD_{50}) and only approximately 20% reduced after four month storage under accelerated stability analysis condition (table 3). At the present time various type of adjuvants such as solution, suspension and emulsion systems are used for immunization against snake venom as a antigen delivery system. Above mentioned adjuvants systems create some problems and have low immunostimulating ability. In the other hand, their preparation process is time consuming and costly. Our results suggested that chitosan nanoparticles, which prepared in our study possibly, could be used as an alternative for traditional adjuvant systems for *Echis Carnatus* venom.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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