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Assessment of DNA Damage During Gene Delivery in Freshwater Prawn by Chitosan Reduced Gold Nanoparticles

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The increasing application of nanoparticles both in industries and in agricultural fields has led to its accumulation in the aquatic ecosystem through water run-off. Insights into the validity of safer nanoparticles such as gold and chitosan are fairly established. However, its effect on aquatic invertebrates has been less studied. The present study was aimed to study effects of chitosan reduced gold nanoparticles (CRGNPs) during green fluorescent protein (GFP) encoding plasmid delivery in giant freshwater prawn, *macrobrachium rosenbergii*. The mean particle size and zeta potential CRGNPs was 33.7 nm and 24.79 mV respectively. Prawn juveniles were exposed to nanoparticles concentrations (10 μg/L, 20 μg/L) of CRGNPs by immersion treatment for a period of 36 hours. GFP was ubiquitously expressed in muscle tissues of prawns. The comet assay indicated dose dependent genotoxicity of CRGNPs in gill, pleopod and muscle tissues which was in conformity with its bioaccumulation pattern *in vivo*. The highest bioaccumulation of CRGNPs was found in Gills, followed by pleopods and least in muscles. Hence, the toxicological potential of CRGNPs to the environment cannot be denied and demands more research on the particular aspect. The doses standardized in the present study would be helpful in safer nano-gene delivery in aquatic invertebrates and development of transgenics employing less cost.

Keywords: Gold Nanoparticles; GFP, Genotoxicity; Bioaccumulation, Comet assay.

In this era of modern biomedical sciences, nanoparticles are being extensively used for applications such as delivery of genes, hormones, vaccines, peptides or proteins intended for therapeutic purpose. Moreover, cell labeling (Bhirde *et al*., 2011), drug targeting (Hans and Lowman 2002), biosensors, and hyperthermia therapy are only a portion of the wide nanoparticle application spectrum (Jeng and Swanson .,2006). Various metal and polymeric nanoparticles (1-100 Nanometers) like gold, Titanium oxide (Ghosh *et al*. 2008; Rather *et al*., 2013)) and Chitosan (Rather *et al*.,2016; Duceppe and Tabrizian 2010)) (Kashyap *et al*.,2015)), poly Lactic-co-Glycolic Acid (PLGA)(Li Y-P *et al*., 2001), polyLactic Acid (PLA)(Kumari *et al*.,2010), poly-å-caprolactone (PCL)(Tang *et al*., 2014)) and gelatin etc.are used for the above purposes.(Soppimath *et al*., 2001; Mahapatro A and Singh DK . 2011). These nanoparticles are being used mainly because of their unique properties such as electrostatic binding, higher intracellular uptake, convenient release profiles and better encapsulation efficiency (Shan *et al*.,2012). In

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mammalian systems such as humans, rats and mouse models, nanoparticles are being used for cancer treatments, targeted gene integration and generation of immunogenic potential(Roy *et al*. 1999). Various non-mammalian and vertebrate models such as zebrafish, black seabream, are also being studied for efficient gene/drug delivery and payload efficiency using nanoparticles(Li *et al*., 2013; Sharif *et al*.,2012)Despite the enormous research done in nanoparticle applicability, little is known about its ill effects to aquatic animal models. The nanoparticles are reported to be genotoxic to host DNA , alters nucleic acids, causes mutations and random inactivation of genes (Shinde *et al*., 2012). Commonly used nanoparticles such as Titanium oxide, silver nanoparticles, carbon nanotubes, fullerenes are responsible to produce chronic cellular damage, apoptosis, genotoxicity and bioaccumulation (Lai *et al*.,2008 ; Chang *et al*.,2012 ;Zhu *et al*.,2016;Cheng SH.,2012) has been recently documented. A number of non-mammalian animal models such as zebrafish have been used to assess the risk associated with nanoparticles on human health (Asharani *et al*; 2008)).Even the widely used gold nanoparticles are biocompatible and believed to be non-toxic, but , but several studies have indicated its semi lethal effects such as lipid peroxidation, cytotoxicity and oxidative stress .(Tedesco *et al*.,2010 ; Pan *et al*., 2007 ; Freese *et al*., 2012 Sharma *et al*.,2016).

In the present aquaculture scenario, the emerging use of nanoparticles for maintaining water quality(Pradeep T and Anshup.,2009),sedimentation of algal biomass (Xu *et al* ., 2011), growth promoting and gene delivery for production of transgenic is taking a step forward. Nanotechnology based DNA vaccines are being developed against White Tail disease in shrimps (Ramya *et al*., 2014), but such treatments may leave behind traces of nanoparticle residues which may find its way to the biological systems. When released as effluents into the aquatic environment, the metal nanoparticles may accumulate in invertebrates and can enter the human food chain. (Baun *et al*.,2008 ;(Judy *et al*.,2011).Since this nanotoxicity is size and dose dependent, it becomes mandatory to formulate the safe dose of nanoparticles before delivering it in farmed conditions (Rather *et al*., 2013).

The giant freshwater prawn (*Macrobrachium rosenbergii*) is a commercially important species for aquaculture and is recently being used in various genetic improvement programs. Very scarce literature is available regarding gene delivery in this species (Ramya *et al*.,2014) and hence, this species was considered as an aquatic invertebrate model to test the possibility of standardization of safer nano-gene delivery doses for transgenic production.

The CRGNPs are reported to be nontoxic in the case of mammalian systems (Pokharkar *et al*., 2009; Bhumkar *et al*.,2007; Stefan *et al*.,2013) but the toxicity potential of these is yet to be established in non-mammalian aquatic invertebrates. Another reason for considering CRGNPs includes the fact that chitosan acts as a penetration enhancer (Bhumkar *et al*.,2007) and hence is helpful in gene delivery practices. As chitosan imparts positive charge to the gold nanoparticles, it has better electrostatic affinity towards the negatively charged DNA/Plasmid. The present study aims to demonstrate Chitosan reduced gold nanoparticles as gene delivery vehicles in *M. Rosenbergii* and its possible effects on cells as well as genetic gradients.

MATERIALS AND METHODS

Chemicals

For the synthesis of CRGNPs, Gold chloride, Chitosan (degree of deacetylation 90%) were procured from Sigma Aldrich (St. Louis, MO) company. For comet assay, Dimethyl Sulphoxide (DMSO) and Low Melting Point Agarose (LMPA) from SRL (Mumbai, India) company were used. Triton X-100 (Sigma) for comet assay and other laboratory wares were obtained from Tarsons (GIBCO, BRL, UK). All other chemicals were of analytical and molecular biology grade. Plasmid PDB402 having 367 bp GFP (Green Fluorescent Protein) gene with CMV (Cytomegalovirus) promoter was procured from Central Institute of Fisheries Education (CIFE), Mumbai,India.

Procurement of animals

Juvenile prawns were collected from a local prawn hatchery at Bharuch, Gujarat, India and kept in FRP (fiber reinforced Plastic)

tanks at the density of 10 prawns/tank of 50 litre capacity in the wet lab of Central Institute of Fisheries Education, Mumbai, India. They were acclimatized to hatchery conditions for two weeks before commencement of the experiment. The water quality parameters were checked fortnightly following APHA guidelines (APHA,1999). The photoperiod maintained in the tank was 12h light/ 12 h darkness. The research undertaken complies with the current animal welfare laws in India. The care and treatment of animals used in this study were in accordance with the ethical guidelines of ICAR-Central Institutute of Fisheries Education, Mumbai,India. As the experimental animal *Macrobrachium rosenbergii* is not an endangered shellfish, the provisions of the Govt of India's Wildlife Protection Act of 1972 are not applicable for experiments on this shellfish.

Preparation and confirmation of CRGNP-plasmid conjugates

CRGNPs were prepared by Turkevich *et al*. method (Turkevich *et al*.,1951) with slight modification. 100 ml of aqueous solution of chloroauric acid (HAuCl 4) of 8mM (4ml) concentration was added to 10 ml of chitosan solution (6.92mg/ml) prepared in 1% acetic acid and heated for 15 min to reduce the chloroauric acid which yields a ruby-red solution. The ruby red colored solution formed is the indication of formation of gold nanoparticles.

For preparing CRGNP- plasmid conjugates, 1 ml of CRGNP solution was dissolved in 9 ml triple distilled water and topped the volume up to 10ml (Rajeshkumar *et al*.,2009). Different concentrations of plasmid solutions (50ng/il, 100ng/il) were prepared by dissolving in 50mM sodium sulfate buffer separately followed by heating at 55^0C. Both plasmid and nanoparticles of variable concentrations were mixed proportions and vortexed on magnetic stirrer at 700 g for 30 seconds. Lastly,the solutions were stored at room temperature. The characterization of resultant nano-plasmid conjugates was done with Beckman Coulter Delsa Nano C- Nano Particle Size Analyzer.for the mean particle size and size distribution. For Scanning electron microscopy, drops of the nanoparticle dispersions were placed on carbon-coated grids. The solution was allowed to air-dry for 1 min. After drying of the samples, they were treated with a high energy electron beam and analyzed using Quanta 200 Environmental Scanning Electron Microscopy (ESEM) system at Icon analytics, Mumbai.

Gel retardation assay

Conjugation of DNA with the CRGNPs was confirmed by centrifuging the conjugate solution at 13,000 g at 48^0 C for 20 min (Wu *et al*.,2010);(Shan *et al*., 2012).After discarding the supernatant, 20 ml autoclaved triple distilled water was added to the pellets. The solution was vortexed for 10 min and then loaded onto 1% agarose gel. The agarose gel was stained with 1ìg/mL ethidium bromide and visualized under UV (Ultraviolet) transilluminator (Syngene, UK).

Dose administration and sampling

The CRGNP doses selected for the study were selected by performing a preliminary test to confirm the rate of mortality in prawns.The CRGNP test concentrations were 10,20,30,40 ug/L of tank water. An immersion treatment to 10 prawn juveniles/ tank was given for 24 hrs. Accordingly, doses which caused least mortality(10ug/L,20ug/L) were selected for final treatement.

The treatment of CRGNP-plasmid conjugates were given (viz. 10ug/L,20ug/L) to animals by immersion treatment in Fibre Reinforced Plastic(FRP) tanks for a period of 36 hours. The animals were stocked at the rate of 6 individuals/Litre in every tank. The animals were

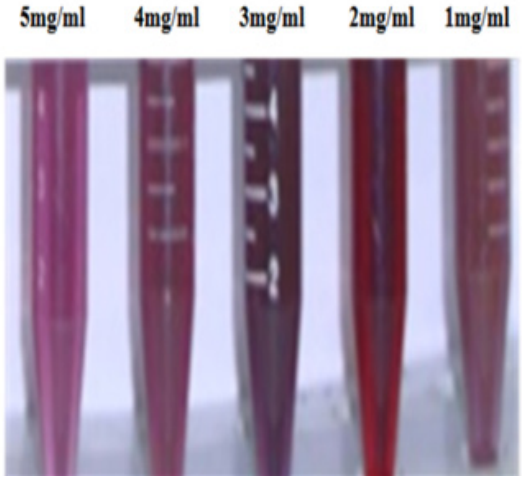

Fig. 1. Chitosan Reduced Gold Nanoparticles of variable concentrations

not fed 24 hours prior to the treatment. Regular aeration was maintained during the treatment. After 36 hours of treatment, the tissue samples were collected from animals by anaesthetizing them with MS222.

Fluorescence microscopy and comet assay

The freshly sampled tissue sections were washed in triple distilled water and the exoskeleton was removed with the help of forceps. The muscle tissues of midbody and near tail end of prawns were then analysed under TCS-SP Leica microscope (Germany) for fluroscence detection. GFP fluorescence absorption peak on 488nm and an emission peak on 509nm was observed.

The comet assay was performed on gills, pleopods and muscle tissues following the method of singh *et al*. [Pavlica *et al*.,2001] with slight modifications. As a positive control, silver nanoparticles (2µg/L) were administered to prawns. For comet assay, the tissues were placed in 1ml of cold Hanks Balanced Salt Solution (HBSS) containing 20mM EDTA in 10% DMSO. The tissues were minced into fine pieces and a suspension was prepared. Slides were prepared in triplicates per concentration and were immersed in cold lysis solution (4oC) at pH 10 for 60 minutes.

After unwinding in the electrophoresis buffer (300 mM NaOH: 1 mM Na2EDTA) at pH13. for 20 min, electrophoresis was conducted at a constant voltage of 25 V and 300 mA at 4oC. Slides were neutralized in 0.4 M Tris having a pH of 7.5 for 5 min and then stained with 75iL Ethidium bromide, cover slipped and immediately analyzed in Zeiss axiophore image analyser. Twenty cells per slide (Pavlica *et al*., 2001) were examined using the software from metasystems

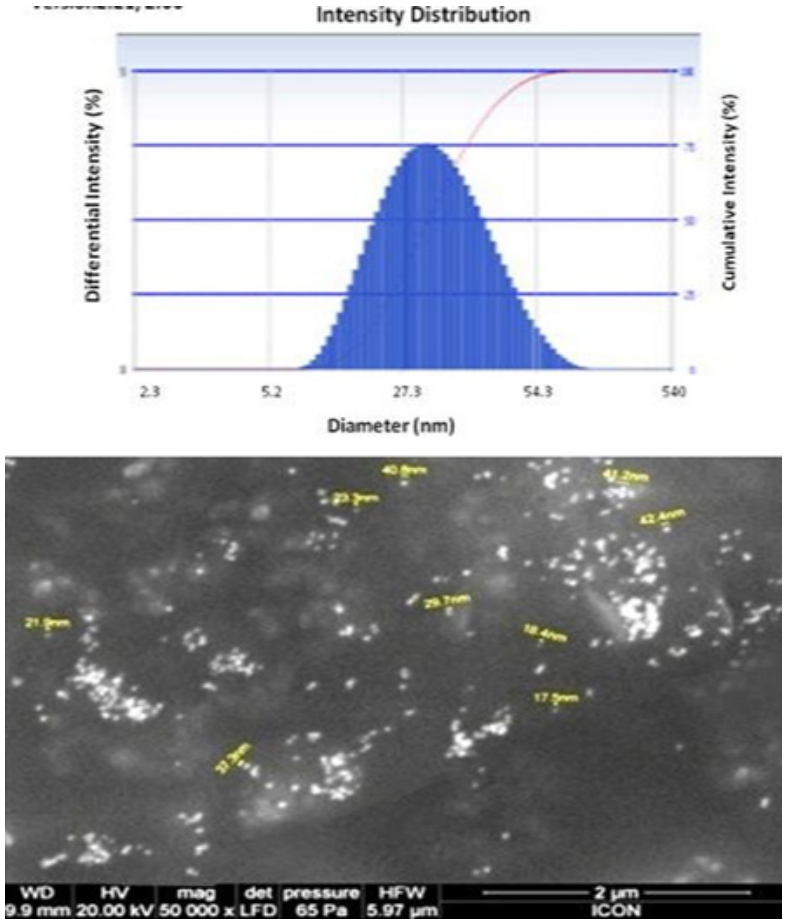

Fig. 2. Particle size distribution and Scanning Electron Microscopy of chitosan reduced gold nanoparticles

(Germany) at the National Center for Preclinical Reproductive and Genetic Toxicology Genetic Toxicology in National Institute for Research in Reproductive Health (NIRRH), Mumbai.

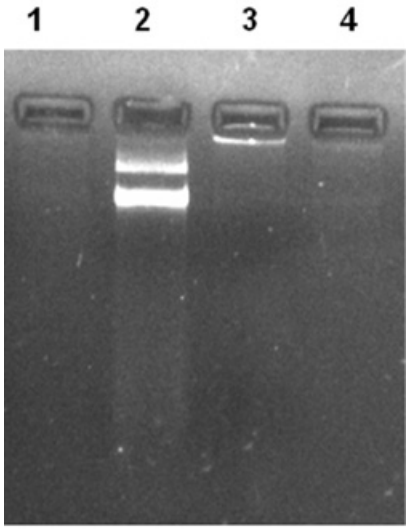

Fig. 3. Gel retardation assay
Lane 1: Blank gold nanoparticles,
Lane 2: Blank plasmid,
Lane 3: DNAse treated plasmid GNP solution,
Lane 4: DNAse treated blank plasmidl

Bioaccumulation studies by Graphite Furnace-Atomic Absorption Spectroscopy (GF-AAS)

GF-AAS was performed on Gill, muscle and pleopod tissues following the method of kehoe (Kehoe*et al* (1988)) with some modifications. The tissues were wet digested using concentrated supra-pure trace-metal free HNO3 (Nitric acid) followed by Conc HClO4 (Perchloric acid). The dried residue was dissolved in 5ml 0.1M HNO3. GF-AAS was performed using a Perkin–Elmer Model-800 atomic absorption spectrophotometer with a Perkin–Elmer HGA-600 graphite furnace. For the analysis graphite tubes were used. After digestion, the sample was diluted with 0.25N supra-pure nitric acid @ 1:2 v/v, prior to analysis. For calibration of the equipment, elemental gold standard (Merck) was used(Lasagna *et al*., 2010).

Data Analysis

The results were analyzed by one-way ANOVA and the comparisons of mean values were carried out by Duncan's multiple range test (DMRT). Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL). All the data analyses were expressed as a mean ± standard error.

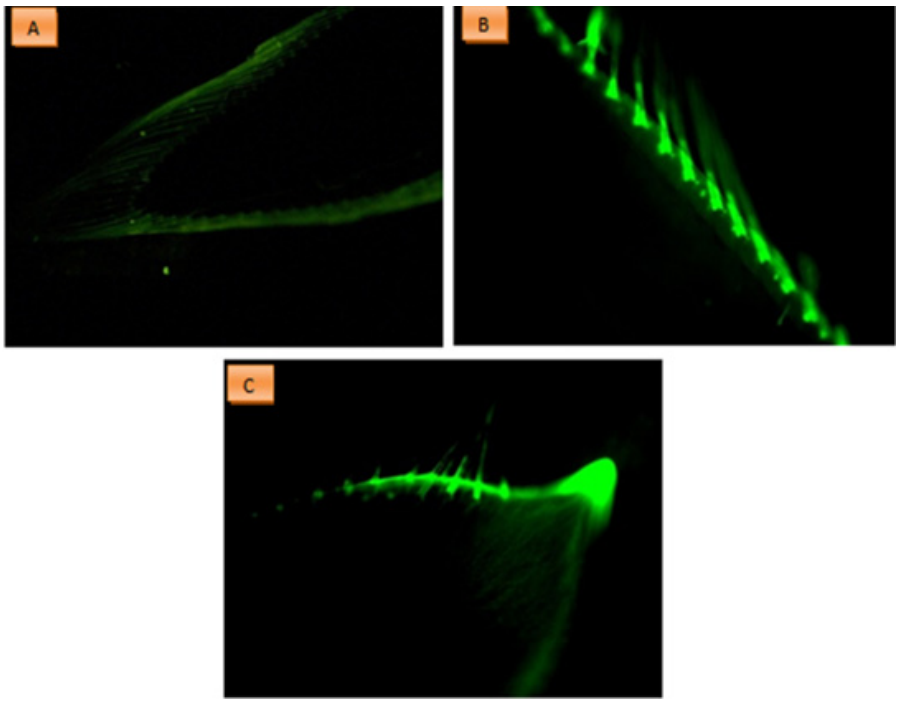

Fig. 4. GFP gene expression in treated animals by fluorescence microscope A: Gills B: Pleopods C: Muscles

RESULTS

Different concentrations of CRGNPs showed color variations ranging from bright red to magenta color (Fig 1). The mean particle size ,polydispersity index and zeta potential of CRGNPs was found to be 33.7nm,0.292 and 24.6mV respectively. The results of SEM confirmed that nanoparticles were compact, spherical in structure and well dispersed. (Fig 1 and 2).

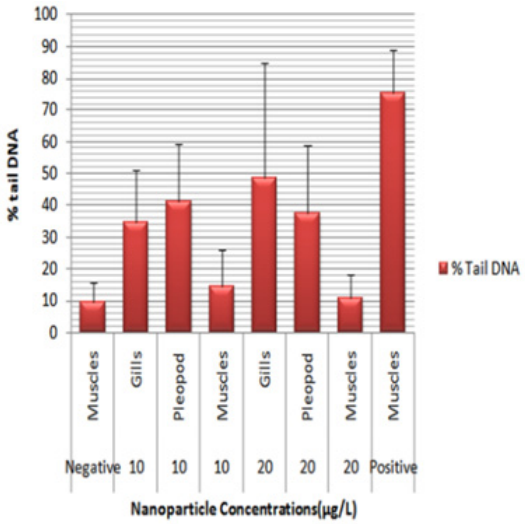

Fig. 5. Percentage of tail DNA in various tissues

The conjugation of plasmid with CRGNPs was confirmed by gel retardation assay. Blank CRGNPs and naked plasmid DNA (pDB 402) was used as controls. The CRGNP- plasmid nanoconjugate did not migrate in the gel. On the other hand, control DNA showed migration from the well (Fig 3). CRGNPs devoid of plasmid did not show any fluorescence in the gel. Further, the bands developed by the CRGNP- plasmid nanoconjugate were less bright than the naked DNA indicating encapsulation of plasmid by CRGNPs.

The intracellular distribution of CRGNP- plasmid nanoconjugates after 36 hours of exposure was detected mostly in gill lamellae (Fig 4a), Pleopods and uropod muscles (Fig 4b, 4c) suggesting the protein expression of the transfected GFP gene. There was no significant difference between GFP expression intensities of samples

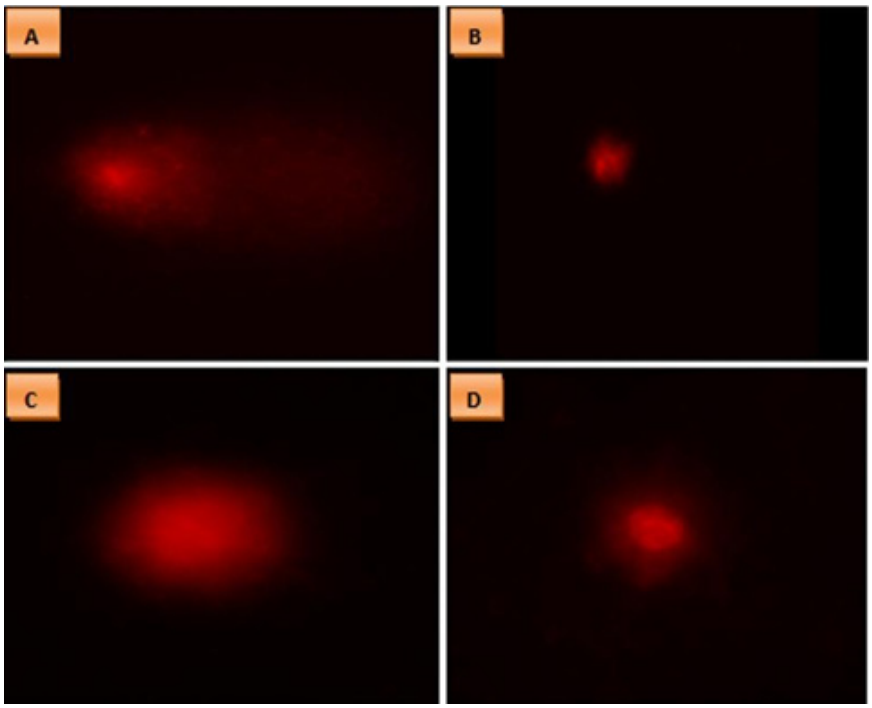

Fig. 6. Genotoxicity caused by Chitosan Reduced gold nanoparticles (Comet assay), **a:** Positive control , **b:** 10 ìg/L, **c:** 20 ìg/L **d:** Negative control. DNA damage is visible in positive control(Silver nanoparticles) while tail length shows concentration dependant damage. No DNA damage is present in negative control shown by absence of tail

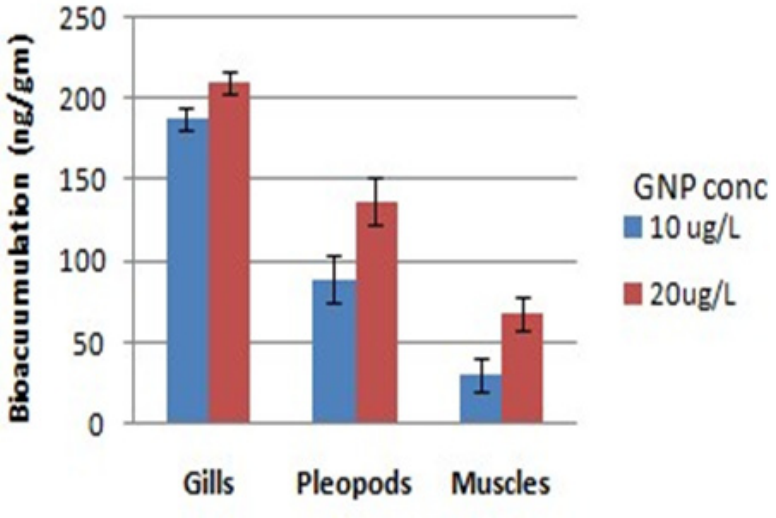

Fig. 7. Concentration dependant (10ug/L and 20ug/L)bioaccumulation of chitosan reduced gold nanoparticles(GF-AAS). Least bioaccumulation is evident in muscle tissues and highest in gills

administered with variable CRGNP- plasmid nanoconjugates concentrations.

Results of cell viability assay showed no significant change in viability compared to Control cells. The % tail DNA content (±S.D) at 10ìg/L was 34.7±16.3% for gills, 41.37±17.7% for pleopods and 14.39 ±11.5% for muscles (Fig 5). At concentration of20 ug/L, it was 48.536±36.03% for gills, 37.6±21.3% for pleopods and 10.9±7.2% for muscles was detected. The negative control group showed very less damaged DNA content in tail DNA(9.75±6.1%) while the positive control (2ug/L silver nanoparticles) it was highest (75.12± 13.8) confirming heavy DNA damage (Fig 6) .

GF-AAS study showed maximum accumulation of CRGNPs in gill tissues of the treated due to maximum exposure to tank water containing nanoparticles.

DISCUSSION

The polymer coated metal nanoparticles are becoming popular as they are known to have less toxicity (Castillo *et al*., 2008 ;Singh *et al*., 2009). The unique features of CRGNPs are tunable core size, large surface to volume ratio, monodispersity and easy functionalization with many biomolecules (Shan *et al*., 2012; Zhang *et al*., 2010). The mean particle size of gold nanoparticles was found to be in the size range as previously reported in studies. Many authors have reported size of the gold nanoparticles ranging from 20 nm to 450nm (Gan *et al* .,200; Li *et al*.,2010). Analysis of gold nanoparticles by polydipersity index suggested narrow range of particle size distribution. Nanoparticles had higher value of zeta potential which showed moderate stability as zeta potential above 30 mV are considered to be more stable (Torchilin VP.,2007) (Fig 1). CRGNPs were synthesized using Chitosan which acted as a reducing agent and provided the positive charge to nanoparticles. The overall positive charge on CRGNPs was evident in Zeta potential measurement and also confirmed the presence of a Chitosan coating over gold nanoparticles. GNP concentrations (10µg/L, 20µg/L) were administered to the animals to ascertain toxic effect on various tissues. Concentration above 80µg/L was found to be toxic by initial trials carried out in our lab. (Li *et al*.,2010) found gold nanoparticles toxicity at the dose of 15µg/L during immersion treatment in *Daphnia magna*. Immersion treatment facilitated even distribution of GNPs in the solution.

Conjugation of plasmid DNA to CRGNPs was the deciding factor during the study. Since the

conjugation could be detected as soon as the buffer containing DNA was added to the nanoparticle solution as evidenced by the change in coloration. The conjugated solution looked purplish in color suggesting increased particle size and increased light scattering resulting in red-shift effect (Huang X and El-Sayed M 2010). When the gel retardation assay for conjugated nanoparticles was carried out, the DNA-nanoconjugates could not migrate in the gel as the neutralization of the negatively charged phosphate group in DNA with the positive charge of nanoparticle obstructed its migration (Wu *et al*.,2010).This confirmed the conjugation of DNA with nanoparticles. Lesser fluorescence in DNA-nanoconjugates was mainly due to fluorescence quenching by gold nanoparticle and increase in publication in the nano-conjugated sample (Kainthan *et al*.,2006).

Consequently, the nanoconjugates after administration to experimental animals via immersion treatment, may possibly escape the endo-lysosomal sections and entered the cytoplasm. Upon entering in endosomes, the proton sponge effect of nanoparticles caused osmotic swelling and subsequent endosomal rupture. This facilitated the escape of DNA-gold nanoconjugate into the cytoplasm and subsequent integration in the nuclear DNA (Guo *et al*.,2010 ;Benjaminsen *et al*.,2012).

Successful delivery of nanoparticles also brings with it the chances of toxic effects.(Li *et al* ., 2010 ; Nandanpawar *et al*.,2013) has reported that the toxicity of silver nanoparticles ranged from 3-4 µg/L in *Daphnia magna*. (Bar-Ilan *et al*., 2009) exposed transparent zebrafish embryos to colloidal silver nanoparticles at various sizes (3, 10, 50, and 100 NM), at a concentration of 100 or 250 ìM, from 4 to 120 hours post fertilization (hpf). Silver nanoparticles produced almost 100% mortality at 120 hpf. In our experiment, when the prawns were exposed to silver nanoparticles (2µl/L) as a positive control, significant mortality was observed and necrosis was visible. Comet assay for CRGPs showed significant DNA damage at higher dose, i.e. 20 µg/L was found to be significant. Higher percentage of tail DNA was indicative of more DNA damage as a consequence of single and double strand breaks (AshaRani *et al*., 2009). On the contrary (Schulz *et al*., 2012) found no genotoxicity in the trachea of rats at 18 ìg concentrations of GNPs. However, in the present study, dose dependant toxicity of GNPs was evident since the exposure time of animals to the variable concentrations was constant. The similar result was also reported by various researchers (Wiwanitkit *et al*., 2009; Hirn *et al*., 2011). The concentration of gold nanoparticles@10µg/L was less toxic with wide tissue distribution and hence could be considered as a safe dose for using CRGNP as delivery vehicles in biomedical applications like gene and vaccine delivery. Low toxicity may be attributed to the non-toxic polymer Chitosan coating over CRGNPs. (Das *et al*.,2012) established a correlation between the capping material and genotoxicity of Gold Nanoparticles. The highest toxicity was reported in gold nanoparticles capped with aspartic acid treatment, while those coated with bovine serum albumin (GNPB) were most biocompatible.

Our study showed a direct correlation between CRGNP dose and bioaccumulation in the organs like gill, pleopods and muscle (Fig 7). The extent of DNA damage and bioaccumulation of gold nanoparticles in pleopods on an average were lesser than gills (Fig 5,7) which may be due to the hardened cell structure of pleopods. The least bioaccumulation was found in muscle tissues. (Lasagna *et al*.,2010) reported reduced bioaccumulation of GNPs as the dose increased. Our study supported this finding as concentration dependant bioaccumulation was observed (Fig 7). The systemic toxicity of gold nanoparticles in the intermediate size range (18–37 nm) was linked to major organ damage in the liver, spleen, and lungs in mice. The muscles being less exposed to aquatic environment had less chances of damage as the treatment was given by immersing the animals in the nanoparticles suspended water. *In vivo* studies of gold nanoparticles have reported bioaccumulation in important body organs, acute inflammation and apoptosis in the liver (Wiwanitkit *et al*.,2009; Xia *et al* ., 2006). On the other hand, repeated administration of higher dose caused no toxicity of gold nanoparticles in mice (Lasagna *et al*.,2010). The absence of mortality at lower doses may be because of the hardy nature of prawn juveniles as compared to the zebrafish embryos.

CONCLUSION

The present study concludes that gold nanoparticles do not cause significant genotoxicity but is responsible for bioaccumulation at higher concentrations. The toxicologically safe doses identified in this study can be used for various applications like gene and drug delivery. The immersion treatment can be considered as an efficient and convenient method of nanoparticles administration when large numbers of animals have to be treated simultaneously with minimum stress. Though Chitosan nanoparticles and gold nanoparticles are considered as safe in nature, their combined effects may indicate some chemical or biological interactions that are still uncharacterized. Further studies on the interaction of these nanoparticles to different biological systems also needs to be advocated for the development of a better delivery system in terms of safety and efficiency.

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