Does Size Matter? Toxicity Effects of 5-nm and 70-nm Silver Nanoparticles on *Hoplobatrachus Rugulosus* Frog

Sineenat Siri (✉ ssinee@sut.ac.th)

Suranaree University of Technology <https://orcid.org/0000-0002-2740-5724>

Kamchan Bamroongnok

Suranaree University of Technology

Buabarn Kuaprasert

Synchrotron Light Research Institute

Ratima Janthima

Suranaree University of Technology

Sukhum Patakkhinang

Ministry of Agriculture and Cooperatives

Pongrit Krubphachaya

Suranaree University of Technology

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Abstract

With the increased usage of silver nanoparticles (AgNPs), the potential impacts of released AgNPs in the environment are increasingly concerned, especially to natural living organisms. Since the properties of AgNPs significantly depend on their sizes, this work aimed to compare the effects of 5-nm and 70-nm AgNPs on toxicity and bioaccumulation in *Hoplobatrachus rugulosus*, the edible East Asian Bullfrog. The synthesized AgNPs were characterized by X-ray diffraction and selected area electron diffraction analyses. Both 5-nm and 70-nm AgNPs caused mortality, reduced growth, induced abnormal development, generated cellular oxidative stress, and modulated cellular biomolecule pattern of frog embryos, but at different levels. The 5-nm AgNPs caused harmful effects on the frog embryos more than 70-nm AgNPs, likely due to their small size to allow more accessibility into the cell. The mortality effects of AgNPs depended on the concentration, exposure time, and size. The malformation of frog embryos in response to AgNPs-exposure included scoliosis, lordosis, kyphosis, and yolk sac edema. Synchrotron Fourier transformed infrared analyses revealed that 5-nm AgNPs significantly changed the profile of cellular biomolecules in the embryos, indicated by the spectral peaks assigned to lipid, carbohydrates, proteins, and nucleic acids. The bioaccumulation of silvers was dominant in eggs, followed by stomach, liver, kidney, and intestine, respectively, suggesting their translocation via blood circulation. The result of high accumulated silver in eggs and effects on embryonic mortality, growth, development, and cellular changes suggested the potential negative impacts of AgNPs on the sustainability of this frog in the environment.

Introduction

Silver nanoparticle (AgNP) is one of the most used nanoparticles in consumer products for antibacterial applications, which their global market size has been continuously rising each year (Kumar et al. 2021). With a growing number of AgNP-incorporated products, the escalated level of released AgNPs into the environment may occur, which can be during the production, transportation, utilization, and discarding processes. Discharge of AgNPs in wastewater is one of the major routes of their entry to the ecosystem, resulting in the increased detection level of silver in natural water reservoirs (Zhang et al. 2021). The estimated concentration of Ag-based NPs was approximately 1–2 ng/L in the River Isar, Germany. Near wastewater treatment plants, the Ag-based NPs concentrations were even higher at 2.47–69.08 ng/L (Wimmer et al. 2019). Therefore, the bioavailability and toxicity of silver in aquatic animals have been increasingly concerned. In particular, the edible species that nanomaterials can bio-transfer to humans via food webs. Also, the sustainability of the natural aquatic animals is concerned after the uptake of nanomaterials at chronic and acute levels.

Among aquatic vertebrates, fish is the most-studied animal model to investigate the effects of AgNPs, which various laboratory and natural fish species are studied. The biological effects of AgNPs in each fish species depended on their developmental stages, studied environments (pure or natural mimic condition), and experimental conditions (exposure time, dose, size, shape, and coating materials) of tested AgNPs (Kakakhel et al. 2021, Liu et al. 2019). The size of AgNPs is one of the factors influencing

the different physical and biological properties of the nanoparticles. A study of size-dependent effect (4 and 10 nm) of AgNPs to zebrafish embryos suggested higher impacts of 4-nm AgNPs than 10-nm AgNPs for uptake, delayed development (indicated by body length and yolk sac area), and transcription of HIF4 and Pxm2 genes relating to hypoxia and membrane transport (Qiang et al. 2020). Cunningham and colleagues also reported the effects of five different sizes (20, 40, 60, 80, and 100 nm) of polymeric-coated AgNPs on adult and larval zebrafish (Cunningham et al. 2021). The results suggested that the smaller size-AgNPs exhibited the higher percent mortality and sublethal malformation of zebrafish, which might involve the better uptake capability of smaller AgNPs. The malformations included no spontaneous movement, yolk sac edema, pericardial edema, and deformation of the eye, snout, and jaw.

As adverse effects of AgNPs were in different aquatic animal species, alternative to fish, a frog is the other species potentially receiving the negative impacts of AgNPs as its niches are mainly in a water environment. The frog is an excellent natural bioindicator of aquatic pollution, including metals, metalloids, inorganic compounds, and pesticides (Pérez-Iglesias et al. 2021, Zaghloul et al. 2020). Also, some species are a source of edible meat for local people or gourmet meat in several countries (Ribas and Poonlaphdecha 2017). Therefore, the studied effects of AgNPs in edible frogs are not only for understanding the impacts of AgNPs in the amphibians but also potentially suggests the potential harms to consumers due to bio-accumulation and bio-transferring AgNPs via food webs.

Thus far, the studies of the effects of AgNPs on frogs are very few. *In vitro* study, AgNPs could alter the expression of transcripts in T3- and stress-mediated pathways of American Bullfrog (*Rana catesbeiana*) tadpoles (Hinther et al. 2010). AgNPs showed the inhibition of the *in vitro* genomic DNA replication of the *Xenopus laevis* frog via the interaction with the minichromosome maintenance protein complex and inhibition of pre-replication complex assembly (Tao et al. 2021). In the behavior study, AgNPs could cause a low predation rate of the Asian Bullfrog (*Hoplobatrachus tigerinus*) tadpoles against *Aedes aegypti* larva under a sublethal dose-AgNPs contaminated aquatic environments (Murugan et al. 2015). Nevertheless, there are very few reports on the *in vivo* studies of AgNP-effects on frogs. There are only two frog species reported regarding the *in vivo* toxicity effects of AgNPs, which are native in Europe and America; the American claw frog (*Xenopus laevis*) and the marsh frog (*Rana ridibunda*). In *Xenopus laevis* embryos, the chronic sublethal exposure of 10-nm AgNPs resulted in the abnormal development of snout/vent and hind-limb. Also, they induced the interference of the expression of some thyroid hormone-responsive genes during the metamorphosis (Carew et al. 2015). The toxicity effect of AgNPs on the tadpole of *Rana ridibunda* frog depended on the AgNP-synthesis methods, which the chemically synthesized AgNPs were more toxic to the tadpole than the physically synthesized AgNPs as determined by the lethal concentration 50 (LC50) of 0.055 ± 0.004 and 0.296 ± 0.085 mg/L, respectively (Johari et al. 2015). The toxicity effect of AgNPs also relied on the type of coated chemicals on the AgNPs. AgNPs capped with the positively-charged branched polyethyleneimine exhibited more developmental malfunction in *Xenopus laevis* embryos than ones covered with the negatively charged citrate (Colombo et al. 2017).

Since the properties of AgNPs also depend on their size, it leads to the question in this work of whether their sizes have different effects on mortality, growth, development, oxidative stress, and cellular profile of biomolecules in the frog embryos. Also, the biological impacts of AgNPs in the edible species of frogs are still a research gap. Therefore, this work aimed to study the effects of 5-nm and 70-nm AgNPs in the edible *Hoplobatrachus rugulosus* frog on mortality, growth, and lipid peroxidation. Also, the biomolecular pattern of frog embryos affected by AgNPs was analyzed by the synchrotron Fourier-transform infrared (FTIR). The localization of the oral uptake of AgNPs (5 nm) in the adult frog was analyzed by atomic absorption spectroscopy in eggs, stomach, liver, kidneys, and intestine.

Materials And Methods

Chemicals

Butylated hydroxytoluene (BHT) and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Silver nitrate was purchased from QRëC Chemical (Auckland, New Zealand). Trichloroacetic acid (TCA) was obtained from VWR Chemicals (Radnor, PA, USA). All chemicals used were of analytical grade.

Synthesis and Characterization of AgNPs

Small spherical AgNPs were synthesized in the reaction containing gelatin (2 g in 190 mL water), 2 M glucose (20 mL), and 1 M silver nitrate (10 mL), which was incubated at 60 °C for 48 h without light exposure (Darroudi et al. 2011). Large spherical AgNPs were synthesized in the reaction containing 0.1 M AgNO₃ (10 mL), 50 mM sucrose (5 mL), and 0.3 M NaOH (5 mL), which was stirred at ambient temperature for 45 min (Thuesombat et al. 2014). The formation of AgNPs was monitored by measuring the absorbance at 300–900 nm using a UV-Vis spectrophotometer (Analytik Jena, Jena, Germany).

Morphology of the synthesized AgNPs was analyzed by FEI transmission electron microscope (TEM; Hillsboro, OR, USA). The suspension of AgNPs was dropped on a carbon-coated copper grid and dehydrated at ambient temperature before observing under TEM. The average particle size and size distribution were determined from 150 particles randomly picked from TEM micrographs using the Image J software (NIH, Bethesda, MD, USA). For characterization, the selected area electron diffraction (SAED)-TEM analysis was conducted by the conventional transmission mode of TEM operating at 200 kV. The nanocrystal nature of AgNPs was determined using a Bruker X-ray diffractometer (XRD; Bremen, Germany). The suspension of synthesized AgNPs was deposited on a glass slide and dehydrated before XRD analysis. The diffracted intensity of Cu K α radiation (λ = 1.54 Å; 50 kV, and 40 mA) was measured in the 2-theta range of 30°-80°.

Effects of AgNPs on Mortality and Growth of Embryos

The experiments that involved animals in this project were approved by the Animal Ethics Committee, Suranaree University of Technology, Thailand (No. 12/2558). The frog embryos were provided by the

Nakhon Ratchasima Inland Fisheries Research and Development Center, Thailand. The development of *Hoplobatrachus rugulosus* frog embryos was studied in a time course of 28 h at 28 °C for 24 h with a photoperiod of 12:12 h of light and dark cycles. The developmental stages of the frog embryos were identified using the Gosner system (Gosner 1960). The toxicity test was carried out by incubating the 3-h stage embryos in pond water (50 mL) containing different concentrations of AgNPs (1, 10, 50, and 100 mg/L) at 28 °C with a photoperiod of 12:12 h of light and dark cycles. Five plastic cups, of which 50 embryos were per cup, were used per treatment, and the data were represented as the average data and standard deviations. The control condition was the embryos incubating in pond water without AgNPs. The mortality rate was calculated as a percentage of a cumulative number of dead frog embryos compared with the total number at 6, 12, 18, and 24 h. Embryonic growth was determined by the lengths of the developed frog embryos, which were measured at 6, 12, 18, and 24 h from the taken images using the Image J software (NIH, Bethesda, MD, USA). Malformations of frog embryos were photographed under an AZ100 stereomicroscope (Olympus, Tokyo, Japan).

Lipid Peroxidation

In vitro lipid peroxidation was determined by the method adapted from Sellami and colleagues (Sellami et al. 2017). The frog embryos treated with 5-nm and 70-nm AgNPs for 18 h from the above experiments were used in a lipid peroxidation assay. The embryos (100 g) were homogenized in ice-cold BHT solution (15 mL) before centrifuging at 4,000 $\times$ g for 30 min at 4 °C. The collected supernatant (1 mL) was mixed with 2 mL of TCA-TBA-HCl reagent (15% TCA, 0.375% TBA and 0.25 N HCl). The mixture was incubated at 100 °C for 15 min and cooled down before centrifuging at 10,000 $\times$ g for 10 min at 5 °C. The absorbance of the supernatant was measured at 535 nm. The lipid peroxidation was determined by measuring TBA-malondialdehyde (MDA) complex, the lipid peroxidation product. The MDA concentration was calculated using the extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. The lipid peroxidation was expressed as nmol MDA per mg protein.

Synchrotron Fourier Transform Infrared (FTIR) Analysis

The synchrotron FTIR analysis in a transmission mode was used to evaluate the effects of AgNPs on the changes of cellular biomolecules. The frog embryos treated with 5-nm and 70-nm AgNPs for 18 h from the above experiments were used for synchrotron FTIR analysis. The frog embryos were embedded in an optimal cutting temperature (OCT) compound and snap-frozen in liquid nitrogen before keeping in a -80 °C freezer. The samples were transversely sectioned (7 mm thickness) using a cryostat microtome (Leica 3050 S, Buffalo Grove, IL, USA) onto an infrared transparent CaF_2 window (13 mm $\times$ 1 mm). Spectral data were collected on the infrared microspectroscopy beamline (BL4.1 IR Spectroscopy and Imaging) at the Synchrotron Light Research Institute (Public Organization, Thailand). Spectra were acquired with a Vertex 70 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) coupled with an IR microscope with an MCT detector cooled with liquid nitrogen over the measurement range of 4000 to 800 cm^{-1} . The measurements were performed in the transmission mode using an aperture size of 10 $\mu\text{m} \times 10 \mu\text{m}$ with a spectral resolution of 4 cm^{-1} and 64 scans. Spectral acquisition and instrument control were performed

using OPUS 7.5 and CytoSpec software (Bruker Optics Ltd., Ettlingen, Germany). The spectra from each cluster of the negative control and treated embryos were analyzed using a Principle Component Analysis (PCA) to distinguish different biochemical components. The spectra were processed using second derivatives and vector normalization via the Savitzky-Golay method (3rd polynomial, 17 smoothing points) and extended multiplicative signal correction (EMSC) via the Unscrambler X 10.5 software (CAMO, Oslo, Norway). The score (3D) and loading plots were used to represent the different data groups and relations among variables of the data set, respectively.

Silver Accumulation

This study was approved by the Animal Ethics Committee, Suranaree University of Technology, Thailand (No. 12/2558). The 1-year-old frogs were provided by the Nakhon Ratchasima Inland Fisheries Research and Development Center, Thailand. They were kept in the outdoor microcosms (one frog per microcosm of 15 cm × 21 cm × 11 cm) under natural photoperiod of approximately 11-h light: 13-h dark and temperature ranged between 28–33 °C for one week to recover from stresses of collection and transportation. The animals were fed once every day with a fish diet (10% of body weight). AgNP-suspension (170 mg/mL) was orally given to frogs (1 mg/g of body weight) using a 10-mL syringe for 10 days. The negative control was to give distilled water to the frogs using a syringe. Three adult frogs were used per experimental condition. After ten days, the frogs were chilled on ice and euthanized by rapid decapitation (Lent et al. 2021). The liver, stomach, intestine, kidney, and eggs were collected and immediately frozen in liquid nitrogen. The samples were digested with nitric acid (0.1 g/mL) with heat until completely dissolved (Uddin et al. 2016). The digested tissues were diluted with 50 mL deionized water before analyzing by atomic absorption spectrophotometer (AAS, Perkin Elmer, Waltham, MA, USA). Independent samples t-test was used for comparing means of the control and sample groups, where significant differences were at $p < 0.05$.

Results And Discussion

Synthesis and Characterization of AgNPs

Two different-sized AgNPs were synthesized using a chemical reduction method. The formation of AgNPs was monitored by the UV-Vis spectrum in a range of 300–900 nm (Fig. 1a). The production of AgNPs was observed by the reaction color that changed from transparent to yellow or yellow-brown and the characteristic localized surface plasmon resonance (LSPR) peak of AgNPs. The narrow spectral curve with the LSPR peak at 410 nm suggested the low size distribution of the small-sized AgNPs (Janthima et al. 2018). The formation of large-sized AgNPs was determined by the LSPR peak at 430 nm but with a broader spectral curve, suggesting their wider size distribution (Alsammarrarie et al. 2018). TEM micrographs revealed predominantly spherical shapes of both synthesized AgNPs (Fig. 1b). The average sizes of the synthesized AgNPs were 5.6 ± 1.4 and 70.9 ± 24.1 nm, which were referred to as small (5-nm) and large (70-nm) AgNPs, respectively.

The synthesized AgNPs were characterized by SAED-TEM and XRD analyses. The SAED patterns of both synthesized AgNPs were similar (Fig. 2a), which contained bright circular fringes corresponding to the (111), (200), (220), and (311) lattice planes, suggesting the crystalline nature of AgNPs according to the JCPDS No. 04-0783 (Acharya et al. 2021). The XRD analysis is shown in Fig. 2b, in which both AgNPs showed similar results of Bragg reflections with the 2-theta values at 38.1°, 44.3°, 64.4°, and 77.3°. These values corresponded to the (111), (200), (220), and (311) lattice planes of the face-centered cubic structure (fcc) of silver according to the JCPDS No. 03-065-2871 (Alahmad et al. 2021). The SAED-TEM and XRD analyses suggested that the identities of the synthesized nanomaterials were AgNPs.

Mortality Effects of Different Sized AgNPs

The 3-h stage embryos were incubated in pond water containing different concentrations (1, 10, 50, and 100 mg/L) of 5-nm and 70-nm AgNPs at 28 °C for 24 h. Their cumulative mortalities in response to AgNPs were measured at 6, 12, 18, and 24 h. The 5-nm AgNPs caused 100% mortality of frog embryos when incubating at the concentrations of 10, 50, and 100 mg/L for 24, 18, and 12 h, respectively. The mortality effect of 5-nm AgNPs depended on dose and exposure time, shown by the linear regression equations (Fig. 3). The higher mortality rates were detected when embryos were exposed to a greater concentration of AgNPs or longer exposure time. The 70-nm AgNPs also induced mortality of the frog embryos, but at a much lower rate. The highest mortality was 22.4% in the treatment of 100 mg/L AgNPs.

Although the mortality effect of AgNPs on frog embryos is unclear, the toxicity mechanism may be similar to fish embryos. Instead of a protective chorion in fish embryos, frog embryos are protected by jelly layers and porous vitelline envelopes (Sato and Tokmakov 2020). The silver nanoparticles in a size range of 1-20 nm can diffuse through lipid bilayers. However, this diffusion process was inefficient for the nanoparticles at 100 nm and larger (Osborne et al. 2015). Due to their small size, AgNPs may penetrate jelly layers and vitelline envelopes more efficiently than the larger-sized AgNPs. Inside the cells, AgNPs may interrupt normal cellular activities and cause impacts on the viability, growth, and development of frog embryos (Qiang et al. 2020). AgNPs can induce lysosomal membrane destabilization, apoptosis machinery, mitochondrial dysfunction, and reactive oxygen species (ROS). Also, the cellular oxidative stress caused by AgNPs can activate the autophagy process, mitochondrial ROS production, and phospholipase-induced membrane degradation (Quevedo et al. 2021). In general, larger-sized AgNPs have lower efficacy to penetrate through jelly layers and vitelline envelopes and accumulate there. These AgNPs can block the vitelline envelope pores, disrupt membrane transport, and destroy the vitelline envelope. Therefore, embryonic development is disruptive, resulting in embryonic malformation (Wu and Zhou 2012). Also, these accumulated AgNPs around the embryos can damage embryos via a release of Ag^+ , which can feasibly penetrate the vitelline envelope (Quevedo et al. 2021). The acute toxic mechanism of Ag^+ in freshwater fish is well established, in which silver ions can induce a disruption of the Na^+/K^+ -ATPase (NKA) ion channel via the inhibition of Mg^{2+} binding. As a result, Na^+ uptake is reduced, thus causing juvenile death if the concentration of Na^+ is decreased by 30% (Boyle and Goss 2018).

Growth Effects of Different-sized AgNPs

In this work, the embryonic developmental stages of *Hoplobatrachus rugulosus* were studied in a time course of 3 – 28 h after fertilization to hatched tadpoles. Their development stages were classified according to the Gosner method (1960). Fig. 4 is the photographs of embryonic development of *Hoplobatrachus rugulosus*, including late cleavage/blastula stage (GS 9; 3 h), early gastrula stage (GS 10; 5 h), mid gastrula stage (GS 11; 7 h), late gastrula stage (GS 12; 9 h), neural fold stage (GS 14; 11 h), elongation and rotation stage (GS 15; 12 h), neural tube stage (GS 16; 13 h), tailbud stage (GS 17; 14 h), muscular response stage (GS 18; 15 h), gill buds and heartbeat stage (GS 19; 16 h), gill circulation and tail elongation stage (GS 20; 19 h), cornea transparent and mouth opening stage (GS 21; 24 h), and tail fins transparent and fin circulation (GS 22; 28 h). These results suggested that the metamorphosis period of *Hoplobatrachus rugulosus* was faster than *Hydrophylax leptoglossa*, *Microhyla ornate*, and *Xenopus laevis* (Hill et al. 2017, Traijitt et al. 2021). These developmental stages were used to identify the frog development in this work.

The frog embryos were treated with different concentrations (1, 10, 50, and 100 mg/L) of 5-nm and 70-nm AgNPs in a time course of 24 h to study their growth and developmental responses. The growth effect was determined by total length, which measured the embryo length (6 and 12 h) or their body length from snout to tail tip (18 and 24 h) (Phung et al. 2020). Fig. 5a shows the effects of 5-nm and 70-nm AgNPs (50 mg/L) on the body length during embryonic development in a time course of 24 h. The results suggested that 5-nm AgNPs had a more negative effect on the body length than 70-nm AgNPs. At 24 h, the average body lengths of the developed embryos treated with 5-nm and 70-nm AgNPs were reduced for 2.6 and 1.8 folds, respectively. Fig. 5b shows the effects of 5-nm and 70-nm AgNPs at different concentrations on the total length of the developed embryos. The results suggested the impact of AgNPs on a significant reduction of the body length of the frog embryos in a dose-respondent manner. Both AgNPs significantly caused the reduction of body length as compared with the control embryos, but more negative effect by 5-nm AgNPs. Also, malformations of frog embryos treated with AgNPs were observed, including scoliosis (abnormal sideways curvature of the notochord), lordosis (bent tail caused by inward curving of the notochord), kyphosis (exaggerated curvature of the upper notochord), and yolk sac edema (Fig. 5c).

These results revealed that 5-nm AgNPs caused more negative effects on the growth of the frog embryos. The growth reduction of the embryos treated with AgNPs could possibly cause by the interaction of AgNPs or the release of Ag^+ with DNA, resulting in disruption of DNA replication, destruction of DNA, and induction of cell apoptosis (Wang et al. 2020). Also, they could affect signaling cascades (such as mitogen-activated protein kinases) that regulate cell proliferation and development, resulting in growth retardation (Zhang et al. 2021). The effects of AgNPs on growth retardation and developmental abnormality might be similar to those in fish embryos via the induction of membrane-pore blockage and reduction of the oxygen exchange rate (Qiang et al. 2020). Also, the delayed growth of frog embryos might result from the toxicity of the releases of Ag^+ via a down-regulation of some genes relating to metabolic rates, ATP synthesis, and ribosome biogenesis (van Aerle et al. 2013). The detected

malformation included scoliosis, lordosis, kyphosis, and yolk sac edema. A group of *Bmp genes* played crucial roles in the early development stage of vertebrate embryos (Suzuki et al. 2017). The interference of these gene expressions might be a cause of abnormal development in AgNP-treated embryos. Although the growth retardation and malformation of frog embryos treated with 70-nm AgNPs were lower than 5-nm AgNPs, this effect might have an indirect impact on the frog survival due to the disadvantages of food and mating competition.

Oxidative Stress in Response to AgNPs

The lipid peroxidation was determined in the frog embryos treated with 5-nm and 70-nm AgNPs at 1, 10, 50, and 100 mg/L for 18 h to reveal the oxidative damage effect of AgNPs on the cellular membrane. The lipid peroxidation was expressed as nmol MDA per mg protein. Fig. 6 shows the MDA levels of the frog embryos treated with 5-nm and 70-nm AgNPs as compared with that of the control embryos. The results revealed that 5-nm AgNPs caused the significantly increased level of MDA in dose-response, and higher than those in the embryos treated by 70-nm AgNPs. At 100 mg/L, for example, the MDA level of the embryos treated with 5-nm AgNPs was approximately 2.7 folds greater than those treated with 70-nm AgNPs. These results also suggested that 5-nm AgNPs caused more toxicity to the frog embryos than 70-nm AgNPs. It was likely that 5-nm AgNPs might penetrate into the cells more efficiently, thus causing greater cellular oxidative stress as seen by the significantly increased level of MDA (the end product of lipid peroxidation) (Mas-Bargues et al. 2021). One possible action of AgNPs to induce oxidative stress is proposed via the ROS-induced peroxidation of the oxidized lipid cell membrane, which disturbs the membrane fluidity and disrupts cell membrane integrity (Yao et al. 2019).

Synchrotron FTIR to Analyze Cellular Molecules

The molecular changes of frog embryos treated with 5-nm and 70-nm AgNPs for 18 h were analyzed by synchrotron FTIR. The spectra data analyses of the head and body regions of the frog embryos were also compared. Fig. 7 illustrates the synchrotron-FTIR spectral analysis of 5-nm AgNP-treated, 70-nm AgNP-treated, and control embryos in head and body regions. The absorption spectra in the wavenumber 3000–1000 cm^{-1} revealed the changed biochemical profiles of lipids, proteins, carbohydrates, and nucleic acids, resulting in distinct separation of the 5-nm AgNP-treated, 70-nm AgNP-treated, and control embryos (Fig. 7a). These results also supported that 5-nm AgNPs exhibited a higher effect than 70-nm AgNPs at a cellular level. FTIR spectral data of both head and body regions were quite similar. From the FTIR spectra, the lipid profiles were indicated by stretching vibrations of asymmetry CH_3 of lipids (2970–2916 cm^{-1}), symmetric stretching vibration of CH_2 of acyl chains (lipids) (2855–2850 cm^{-1}) (Talari et al. 2017), and ester group ($\text{C}=\text{O}$) vibration of triglycerides (1750–1745 cm^{-1}) (Nandiyanto et al. 2019). The α -helical and β -sheeted conformations of amide I were the spectral peaks at 1657–1650 cm^{-1} and 1635–1625 cm^{-1} (Nandiyanto et al. 2019). The indication of carbohydrates was via the O–H in-plane bend of primary or secondary alcohol (1350–1260 cm^{-1}) (Nandiyanto et al. 2019), C–O asymmetry stretch of ketone (1150–1145 cm^{-1}), C–O–C asymmetry stretch of ether (1120–1094 cm^{-1}), and C–O stretch of primary

alcohol ($1070\text{--}1050\text{ cm}^{-1}$) (Talari et al. 2017). For nucleic acid analysis, the spectral peaks at $1265\text{--}1201\text{ cm}^{-1}$ and $1099\text{--}1066\text{ cm}^{-1}$ corresponded to the asymmetric and symmetric PO_2^- stretching vibrations (Talari et al. 2017).

Fig. 7b shows the principal component analysis (PCA) plots. The PCA analyses demonstrated that the PC-1, PC-2, and PC-3 provided the best visualization of separated clusters of biochemical changes among three groups of the samples; 5-nm AgNP-treated, 70-nm AgNP-treated, and control embryos. The PCA score plot of the head region was sufficient to separate these three sample groups, which was explained by 37% PC-1 and 9% PC-3. Similarly, the PCA score plot of the body region was separately clustered by 38% PC-1 and 13% PC-2. Fig. 7c shows the first principal component (PC-1) loadings plots that give the main variation between these sample groups. According to the second derivative spectra (Fig. 7d), the separation of 5-nm AgNP-treated, 70-nm AgNP-treated, and control embryos dominantly attributed to lipids ($2960, 2958, 2925, 2923, 2852$, and 1745 cm^{-1}). Also, the separation could assign by proteins (1656 and 1629 cm^{-1}), carbohydrates ($1344, 1147, 1114, 1060$, and 1058 cm^{-1}), and nucleic acids ($1239, 1238, 1089$, and 1087 cm^{-1}). In the head region, the second derivative plot revealed the lipid reduction at a higher level in the frog embryos treated with 5-nm AgNPs than 70-nm AgNPs. These reduced spectral peaks ($2960, 2923, 2852$, and 1745 cm^{-1}) attributed to the lipid reduction. The protein conformational changes, α -helical (1656 cm^{-1}) to β -sheeted (1629 cm^{-1}), were also detected in frog embryos treated with AgNPs of both sizes. The frog embryos treated with AgNPs revealed induced carbohydrate levels indicated by the spectral peaks at $1344, 1147, 1114$, and 1060 cm^{-1} . Similarly, 5-nm AgNPs exhibited more carbohydrate induction as compared with 70-nm AgNPs. The increase and decrease of nucleic acid levels in response to AgNP-treatment were noticed by the spectral peaks at 1238 and 1087 cm^{-1} , respectively.

In the body region, synchrotron-FTIR results were similar to those in the head region analysis. The lipid reduction was detected, as indicated by the spectral peaks at $2958, 2925, 2852$, and 1745 cm^{-1} . For protein analysis, the α -helical and β -sheet structures were present in the embryos treated with 5-nm AgNPs as indicated by the spectral peaks at 1656 and 1629 cm^{-1} . The carbohydrate induction in frog embryos treated with AgNPs was also detected at the spectral peaks of $1344, 1147, 1114$, and 1058 cm^{-1} . The modulation levels of nucleic acids in response to AgNP-treatment were noticed by the spectral peaks at 1239 and 1089 cm^{-1} .

Accumulation of AgNPs in Frog Eggs and Some Organs

The localization of AgNPs in the frog digestive system and the excretory system was analyzed by atomic absorption spectroscopy analysis (Table 1). In this work, 5-nm AgNPs were orally given to the adult frogs ($1,000\text{ mg/kg}$) every five days for 30 days. The levels of silver in the AgNP-treated samples were significantly higher than the control samples at all analyzed tissues. The highest silver level was detected in eggs, followed by the stomach, liver, kidney, and intestine. The detected amount of silver in eggs was as high as $195.55 \pm 0.87\text{ }\mu\text{g/g}$ of wet weight tissue, making the concern about the effects of these silver

on their embryonic development. These results suggested that AgNPs could absorb into the blood circulation and translocate to the other tissues and organs. The localization of AgNPs in the liver and kidney was likely due to these organ functions involving detoxification and excretion of toxic materials. As eggs could store a high level of AgNPs, it raised a concern about the potential effects of AgNPs on the reproductive system and the survival rate of the frog embryos. As a result, at this exposure dose, AgNPs might affect the sustainability of this frog species in nature.

Conclusions

The results of this work revealed that AgNPs of different sizes (5 and 70 nm) had similar effects on survival, growth, development, oxidative stress, and cellular response in the *Hoplobatrachus rugulosus* frog, but at different degrees. The 5-nm AgNPs significantly caused more negative effects on the frog embryos than 70-nm AgNPs, potentially resulting from their effectiveness to enter the cell by size. The higher mortality effects of 5-nm AgNPs were also dose- and time-dependent. The reduction of body length during embryonic development was significant in AgNP-exposed embryos, and the 5-nm AgNPs caused a higher negative effect of body length than the 70-nm AgNPs. The malformation of the survived embryos exposure to AgNPs included scoliosis, lordosis, kyphosis, and yolk sac edema. Synchrotron Fourier transformed infrared analyses supported that 5-nm AgNPs modulated the regular patterns of cellular biomolecules in the embryos more than those of 70-nm AgNPs. The results also indicated that the dominant bioaccumulation of AgNPs was in eggs after the adult frogs obtained AgNPs via feeding. High accumulated AgNPs in eggs might cause mortality, retarded growth, abnormal development of the eggs. As a result, it might cause a potential negative impact on the sustainability of this frog in the environment.

Declarations

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Conflict of interests The authors declare that they have no competing interests.

Ethics approval The experiments involved animals in this project were approved by the Animal Ethics Committee, Suranaree University of Technology, Thailand (No. 12/2558).

Consent to participate All authors informed consent to participate in this paper.

Consent for publication All authors informed consent to publish this paper.

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Tables

Table 1
Accumulation of AgNPs in adult frogs.

Tissues	Detected amounts of silver (µg/g)	
	Control frogs	Treated frogs
Liver	0.95 ± 0.13	10.30 ± 0.45*
Stomach	1.87 ± 0.21	91.45 ± 0.37*
Intestine	0.61 ± 0.17	5.97 ± 0.32*
Kidney	0.53 ± 0.11	6.02 ± 0.24*
Egg	0.23 ± 0.11	195.55 ± 0.87*
N = 3, * $p < 0.05$		

Figures

Figure 1

UV-Vis spectra (a) and TEM micrographs (b) of the synthesized AgNPs

Figure 2

SAED-TEM (a) and XRD (b) analyses of the synthesized 5-nm and 70-nm AgNPs

Figure 3

Mortality effects of 5-nm and 70-nm AgNPs on frog embryos at 6, 12, 18, and 24 h. Insets show the linear regression of the mortality of frog embryos in response to 5-nm AgNPs. Independent sample t-test was used for group comparisons at each time point

Figure 4

Identification of embryonic developmental stages of *Hoplobatrachus rugulosus* according to the Gosner's 46 stages

Figure 5

The effects of 5-nm and 70-nm AgNPs (50 mg/L) on the body length of frog embryos in a time course of 24 h (a). One-way ANOVA with post-hoc Tukey HSD test was used to compare the data at the same time points (a). The effects of different concentrations (0, 1, 10, 50, and 100 mg/L) of 5-nm and 70-nm AgNPs on the body length of frog embryos at 18 h (b). One-way ANOVA with post-hoc Tukey HSD test was used to compare the data at different concentrations of AgNPs (b). The detected malformation of frog embryos treated with AgNPs (c). Ctrl, control; ky, kyphosis; lo, lordosis; sc, scoliosis; and yse, yolk sac enema

Figure 6

Lipid peroxidation of the frog embryos treated with 5-nm and 70-nm AgNPs at 1, 10, 50, and 100 mg/L for 18 h. One-way ANOVA with post-hoc Tukey HSD test was used to compare the data

Figure 7

Synchrotron FTIR analyses of frog embryos treated with 5-nm and 70-nm AgNPs for 18 h, which displayed in the absorption spectra (a), PCA-score plots (b), PC-1 loading plots (c), and second derivative spectra (d). The head and body regions of frog embryos were analyzed for comparison (left and right panels, respectively)