Organometallic Folate Gold Nanoparticles Ameliorate Lipopolysaccharide-Induced Oxidative Damage and Inflammation in Zebrafish Brain

Susanta Sadhukhan,∥ Mahammed Moniruzzaman,∥ Subhajit Maity,∥ Sudakshina Ghosh, Arup Kumar Pattanayak, Suman Bhusan Chakraborty, Biswanath Maity, and Madhusudan Das*  

ABSTRACT: Synthesized organometallic gold-based folate nanoparticles (FAuNPs) were characterized, and its defense against lipopolysaccharide (LPS)-induced brain inflammation in Zebra fish was proven. Vitamin entrapment efficiency of these particles was found to be nearly 70%. The in vitro pH-dependent drug release dialysis study of FAuNPs confirmed a slow, sustained, and gradual release of folate for a period of 24 h. Both AuNPs and FAuNPs did not cause any marked changes in food intake, body weight, color, behavioral pattern, blood parameters, and hepatotoxicity. Histology of liver showed no changes between treated and control groups of fishes. The ex vivo study showed significant uptake of FAuNPs to free folate in folate receptor negative Hek293 cells, confirming a strategy to overcome folate deficiency in the brain. Antioxidant status and activities of few crucial brain enzymes were also measured to assess the brain function and found to be returned to the basal level, following FAuNP treatment. The transcription factor NRF2-Keap 1 expression pattern was also noted, and a prominent modulation was observed in the LPS-treated and FAuNP-administered group. Decisive brain enzymes like AChE and Na⁺K⁺ATPase were decreased significantly after LPS treatment, which is restored with FAuNP treatment. Caspases increased sharply after LPS treatment and diminished following FAuNP treatment. We conclude that FAuNP due to its high physical stability and uptake could be utilized against severe brain inflammation, leading to brain injury and neurodegeneration.

INTRODUCTION

Folate is crucial for the proliferation of neural progenitor cells and synaptic activity. Excess blood homocystine is an indicator of folate deficiency. This causes aberrant homocystinylation of neural proteins, which hamper neuronal development.1 Our body is not able to synthesize folate, rather one has to meet up the daily necessities of folate by foods or through chemically synthesized oxidized folate form as vitamin supplements. Therefore, taking folic acid as supplements is the only way to overcome this deficiency and related neuronal disorders.2 Folate is one of the key factors for the regulation of acetylcholine esterase (AChE) and Na⁺K⁺ATPase enzymes that are imperative for the activity of the central nervous system and brain. In many cases, folate deficiency has been observed due to structural and functional abnormality of folate receptors (FRs).3 Nanoparticle-mediated delivery systems often have the prospect to boost bioavailability and therapeutic efficacy of the drug by minimizing the concentration of free drug and reducing its side effects.4,5 Organometallic nanoparticles are small and, therefore, can cross the cell membrane effortlessly independently without any specific receptor or transporter. Metals may sometime lead to toxicity load, but gold is naturally found to be chemically inert and beneficial due to its antioxidant properties.6 We, therefore, designed to develop a gold nanoparticle (GNP) of folate (FAuNP), which could enter into the cell directly irrespective of the receptors.  

In the present study, Zebra fish was treated with lipopolysaccharide (LPS) to develop a state of folate deficiency. LPS is a unique surface molecule of Gram-negative bacteria, which causes enormous hazards for the biological system depending on the species and dose.6,7 Bacterial LPS isolated from Escherichia coli has been used to promote inflammation and immunomodulation in fishes. LPS is the primary component of the outer membrane of Gram-negative bacteria that leads to the production of series of endogenous inflammatory mediators in response to host defense. However,
unrestrained and unwarranted release of inflammatory mediators initiates free radical production that eventually causes oxidative stress.\textsuperscript{8} Excessive free radical accumulation disrupts prooxidant–antioxidant stability and alters the level of antioxidants. Another fundamental factor in the regulation of free radical production and ROS amassing in the brain is xanthine oxidase (XO). XO is noted to be a significant source of superoxide radicals and generator of ROS. NF-E2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (Keap1) are imperative factors, which mediate transcription of numerous stress genes including most of the antioxidative genes during acute and chronic stresses.\textsuperscript{9} Under invariable stress, all these genes are positively regulated by NRF2 and protect cells from cell death.\textsuperscript{10} Keap1 plays a key role in stress, all these genes are positively regulated by NRF2 and disrupts prooxidant genes during acute and chronic stresses.\textsuperscript{9} Under invariable (Keap1) are imperative factors, which mediate transcription of antioxidants. Another fundamental factor in the regulation of degeneration and maintenance of normal brain function. Results of this study would be useful in scheming an effective management system to overcome predicament related to folate deficiency and brain injury.

2. MATERIALS AND METHODS

2.1. Materials. Folic acid, gold chloride, dialysis sacks, Dulbecco’s modified Eagle medium (DMEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, USA. Sodium citrate, potassium bromide (KBr), chloroform, hematoxylin, eosin, and paraffin wax were purchased from Merck, Germany. Glucometer (AccuCheck), cholesterol, and triglyceride kits were obtained from Span Diagnostics, Mumbai, India. Alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) kits were obtained from Robonik-prietest-Clinical Chemistry Reagents, Mumbai. All other reagents were of high analytical grade.

2.2. Synthesis and Characterization of Folate-GNPs. GNPs were synthesized by the sodium citrate capping method.\textsuperscript{11} To synthesize AuNPs, 20 mL of 1 mM gold chloride was added to 1% sodium salt of citric acid under boiling environment. After a few minutes, it was observed that the faint yellow solution became beetroot extract color (red wine/mixture A). The synthesized nanoparticles were purified by spinning at a high gravity of 12,500 for 15 min. After this, repetitive washing was done with Millipore water to remove untagged citrate.

For the preparation of FAuNPs, 5 mg of folic acid (folate allowed to soluble in diluted dimethyl sulfoxide) was added to that wine red mixture A under boiling conditions again. The final concentration of folate was 0.25 mg folate/mL of nanoparticles. Lastly, the FAuNPs were suspended in Millipore water, spun (12,500 g) for three times, and allowed to freeze dry overnight (preparation for dry nanoparticles whenever required). To study the efficacy of folate attached on the surface of citrate-capped AuNPs, UV–vis spectrophotometric analysis was carried out. The concentration of folate was estimated before and after nanoparticle entrapment from its molar extinction coefficient at 283 nm. After spinning of FAuNP solution at 12,500g for 20 min, the remnant folate in the supernatant was analyzed by a Jasco V630 UV–vis spectrophotometer. The particle size of nanoparticles (FAuNPs) was assured by dynamic light scattering (Zetasizer Nano ZS Malvern Instrument, UK). Autocorrelation was measured at normal room temperature.\textsuperscript{12} Zeta potential was also done to measure surface charge. For the morphological structure study by transmission electron microscopy (TEM Techna SEII), 10 μL of FAuNP solutions was taken on carbon-coated copper grids. It was allowed to dry for few minutes and then loaded in the TEM chamber after phosphotungstic acid staining.

Infrared spectra of three samples, namely, free folate, AuNPs, and FAuNPs were scanned in potassium bromide (KBr) pellets in the frequency band of 4000–400 cm\textsuperscript{-1} at normal room temperature using Bruker Fourier transform infrared (FTIR) (Model-Alpha E, Germany).

The folate discharge study was analyzed using a dialysis method.\textsuperscript{12} Freshly synthesized FAuNPs and free folate were positioned within the dialysis sack and then dipped in 50 mM sodium phosphate buffer having pH 7.4 and acidic sodium citrate buffer pH 3.0, respectively. Samples were withdrawn at 15 min interval, and the concentration of released folate was estimated by a UV–vis spectrophotometer at a fixed wavelength. % of released vitamin was calculated by

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\text{vitamin release (%) = released vitamin/total vitamin} \times 100
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2.3. Ex Vivo Studies. HEK293 and WI38 cell lines were obtained from National Center for Cell Sciences, Pune, India. The cultures were grown in an incubator at controlled environment of 37 °C and 5% CO\textsubscript{2} in DMEM. They were grown until 70–80% confluency at 10\textsuperscript{4} cells/plate. For cytotoxicity assay, the cells were seeded in 96-well plates at 10\textsuperscript{4} cells/well. After 3 h incubation with varied concentrations of FAuNPs (0–50 μg/mL), cell viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).\textsuperscript{13}

2.3.1. Uptake Study by Fluorescence Activated Cell Sorter. For initiating the treatment, the medium was reinstated with starvation medium containing varied concentrations of FAuNPs (25 and 50 μg/mL). After 3 h of incubation, cellular uptake was estimated by VERSE FACS at a 450 nm emission filter with excitation at 360 nm wavelength.\textsuperscript{14}

2.4. Experimental Model. Zebrafish (Danio rerio) (weighing approximately 8 gm) were housed to first acclimatize under a controlled environment (room temperature: 25–28 °C, 14:10 light/dark rhythm) with a balanced diet (commercially available Tetra bits) and water ad libitum for about 1 week prior to start the toxicity studies.\textsuperscript{15}

2.5. Experimental Design. After 10 days of acclimatization, 30 adult fish were indiscriminately alienated into five different treatment groups as follows: (i) C-control (n = 6), (ii) T-exposed to LPS (n = 6), (iii) C1-control with FAuNP exposure (n = 6), (iv) TN1-LPS-exposed fish treated with FAuNPs for 3 days (n = 6), and (v) TN2-LPS-exposed fish treated with FAuNPs for 5 days (n = 6). The size of the aquarium was 150 × 60 × 50 cm for all the experimental groups. After completion of the experiment, fish were taken out, anesthetized (phenoxy-ethanol; 1:20,000; v/v), and immediately sacrificed. Brain tissues were dissected out immediately and stored at −20 °C. The entire experimental setup has been replicated three times. All fish were fed commercially obtainable balanced diet during the entire experimental period but starved 24 h before sampling. The experiment was conducted under semistatic exposure con-
ditions, and water was renewed daily to remove excess diet and detritus. Temperature, pH, and dissolved oxygen were monitored regularly, and no significant difference between the experimental groups was observed throughout the experiment. The investigation was performed according to the guidelines of the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication no. 85−23, revised 1996) and was also permitted by Institutional Animal Ethics Committee, University of Calcutta (Registration #885/ ac/05/CPCSEA), registered under “Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals” (CPCSEA), Ministry of Environment and Forests, Government of India.

2.6. Toxicity Studies of the FAuNP Treatment. Chronic toxicity studies were carried out with both folate GPNPs (FAuNPs) and AuNPs at a dose of 1 mg kg−1 b w/day for a period of 3 and 5 days consecutively in zebrafish. Experimental fish were primarily divided into two groups (one for 3 days and another one for 5 days), and each group is again subdivided into the following three subgroups (n = 3): subgroup 1 (normal control/NC): placebo, subgroup 2 (treatment I): AuNP (1 mg kg−1 b w/day), and subgroup 3 (treatment II): FAuNP (1 mg kg−1 b w/day) orally. First, on the 3rd day, blood samples (1000 μL) were collected by cardiac puncture under 100% chloroform anesthesia. Heparinized blood was centrifuged (10 min; 2500 g; 4°C) and stored at −20°C for analysis of biochemical parameters. Second, on the 5th day, the same process was followed to obtain serum for biochemical analysis.

2.6.1. Biochemical and Physiological Parameter Test. Different biochemical parameters like glucose, total protein, triglyceride, calcium, and cholesterol were estimated using standard kits (as per the manufacturer’s instructions; Robonik-prietest-Clinical Chemistry Reagents).

Serum was used from the FAuNP/AuNP-treated and NC groups to estimate the hepatic damage markers, namely, ALP and SGPT by using standard kits (Robonik-prietest-Clinical Chemistry Reagents).

2.6.2. Brain Enzyme Assay. Brain tissues were homogenized with Tris-buffered saline (10 mM Tris-HCl, 0.1 mM EDTA-Na, 10 mM sucrose, 0.8% NaCl, pH 7.4) and centrifuged, and the supernatant was used to measure levels of brain enzymes. Na+−K÷ATPase activity was calculated by estimating the liberation of PO4−3 from hydrolytic reaction with ATPase. AChE activity in the tissue homogenates was estimated using a standard kit.

2.6.3. Analysis of Brain Stress Markers. Brain supernatants were used to estimate malondialdehyde (MDA) levels. MDA is the resultant product of lipid peroxidation by thiobarbituric acid-reactive substances. Glutathione (GSH) was spectrophotometrically assessed at 412 nm following a standard method. Superoxide dismutase (SOD) activity was calculated by a calibrated spectrophotometric method through the assessment of O2−-induced NBT reduction. Catalase (CAT) activity was assessed following a standard method. Absorbance was monitored at 240 nm up to 90 s at a regular 15 s interval.

Relative activity of XO was determined using a standard method earlier described. The reaction mixture (1 mM of xanthine as substrate and 50 mM of phosphate buffer) was incubated with the homogenized tissue sample at 37°C for 1 h. The rate of urate production from the breakdown of xanthine was estimated by measuring the absorbance at 290 nm.

2.6.4. Histological and Tissue Architectural Study. For histological analysis, liver tissues were fixed in 10% formaldehyde solution. Fixed liver tissues were dehydrated in graded alcohols, and routine microtomy was carried out for the procurement of 6 mm thick paraffin sections, which were stained with hematoxylin and eosin (H&E) stains and studied under a bright-field microscope (Olympus BX51, Japan). At least 10 indiscriminately chosen mid-sagittal sections were used for the study.

2.7. Immunoblotting. Expression of different proteins in the brain tissue of fish from different treatment groups was calculated through 12.5% Laemmli sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting on a poly(vinylidene difluoride) (PVDF) membrane by the wet electroblotting method. Primary antibodies (Caspase 3/7/Caspase 9/NRF2/Keap1) were obtained from Abcam (Cambridge, MA 02139-1517, USA). The PVDF membrane was incubated with the primary antibody (1:1000 overnight, followed by incubation with the secondary antibody (1:500) for 2−3 h. Analysis of the relative densitometric value was executed to calculate individual band intensity of each immunoblot using Image J Software.

2.8. Statistics. Data belonged to each clusters were analyzed using three separate univariate one way ANOVA. All the values were expressed as mean ± SE, and the significance of the main effects was evaluated through the study of the P>F (P<0.05) value where F values designate the level of significance. The values (n = 6; 3 replicates) were evaluated by a post hoc Tukey test with P-value (0.05) as the threshold.

3. RESULTS

Absorption spectrophotometric analysis showed a peak ~530 nm in the case of AuNPs, but the same peak of FAuNPs undergone a red shift along with an extra characteristic of folate in ~283 nm (shown in Figure 1a). The absorbance experiment was also done to calculate the entrapment efficiency of folate in the folate-GNP complex, as shown in Figure 1b. The absorbance spectra of free folate 0.25 mg/mL [Figure 1b(i)] and folate remnant in the soup after centrifugation of the complex at 12,500 g, indicating tagging of folate on the surface of citrate-capped GNP.
The average diameters of AuNPs and FAuNPs were found to be 30 nm (data not shown) and 56 nm, respectively, as analyzed from DLS (representative of FAuNPs shown in Figure 2a). It was also noted that the surface charge present on FAuNPs was $-21.9$ mV, as measured by the zeta nanosizer (Figure 2b). In the TEM view, FAuNPs appeared to be

Figure 2. (a) Particle size determination of FAuNPs by DLS. (b) Surface charge estimation present on FAuNPs by the zeta nanosizer. (c) Shape and size analysis of FAuNPs by TEM, depicting the structural conformations of the nanoparticles.

Figure 3. (a) FTIR spectra of free folate, AuNPs, and FAuNPs using potassium bromide pellets, indicating the formation of the folate GNP complex. (b) In vitro pH-dependent drug release study at acidic and neutral environments (mean ± SD of three experiments), showing slow, sustained, and gradual release.
spherical in nature (of around 50 nm in size) and dispersed in the microscopic field, as evident and shown in Figure 2c.

3a depicts the Fourier spectra of free folate and both the AuNPs. In both acidic pH 3.0 and neutral pH 7.4, there was a slow and steady release of the vitamin folic acid for 24 h (a chief criterion for every nanoparticles) (Figure 3b).

No statistically significant cellular toxicity was found even at nanoparticle concentrations (50 μg/mL) for Hek293 and WI38 cells at 3 h of incubation (data not shown). The cultured FR α negative (FRα⁻) HEK 293 cell line showed an uptake of free folate, which is very negligible (around 0.2%) at both 25 μg/mL dose and 50 μg/mL dose almost equivalent to normal control (without any free folate treatment), whereas folate-conjugated GNP treatment showed a significant elevation of folate uptake 8.55 and 35.73% at 25 μg/mL dose and 50 μg/mL dose, respectively. The data are shown in Figure 4.

On the other hand, at 25 μg/mL free vitamin dose, the FR α positive WI38 cell line (FRα⁺) showed an uptake of around 1.58%, whereas 50 μg/mL dose exhibited an uptake of approximately 2.02% at 3 h of incubation, but in contrast, the folate-conjugated GNP showed a more elevated amount of uptake at 25 μg/mL dose (4.32%) and 50 μg/mL dose (16.36%) (Figure 5). The HEK 293 FR α negative (FRα⁻) cell line shows no uptake of free folate.

The chronotoxic effects of blank nanoparticles (AuNPs) and folate GNPs (FAuNPs) showed no significant differences in the abovementioned tests in [group 1 (control): NC placebo], [groups 2 and 3 (treatment I): AuNP (1 mg kg⁻¹ b w/day) for 3 and 5 days, respectively], and [groups 4 and 5 (treatment II): FAuNP (1 mg kg⁻¹ b w/day) for 3 and 5 days, respectively] orally treated animals. Glucose/lipid profiles and liver toxicity markers are shown in Figures 6 and 7, respectively.

Histopathological analyses of liver were also done for confirmation. Hepatic sections of [group 1 (control): NC placebo], [group 2 (treatment I): AuNP (1 mg kg⁻¹ b w/day) for 5 days], and [group 3 (treatment II): FAuNP (1 mg kg⁻¹ b w/day) for 5 days] showed almost perfect central vein, healthy sinusoids, and distinct nuclei (Figure 8).

MDA, SOD, CAT, and XO significantly (P < 0.05) increase in the LPS-treated group, while they significantly (P < 0.05) decrease in FAuNP-treated groups in a duration-dependent manner (Figure 9). GSH significantly (P < 0.05) increases in the LPS-treated group and decreases thereafter following FAuNP treatment (Figure 9).

AChE shows a significant (P < 0.05) decrease in the group treated with LPS compared to control in all regions of brain. Fish groups with FAuNP treatment show a significant increase (P < 0.05) in AChE in all regions of brain compared to the group treated with LPS. Na⁺K⁺ATPase shows a significant (P < 0.05) decrease in the group treated with LPS compared to control in all regions of the brain. The D5 group shows a significant increase (P < 0.05) in Na⁺K⁺ATPase in CC and CE compared to the group treated with LPS (Figure 10).

The group treated with LPS, D3, and D5 shows a significant (P < 0.05) increase in caspase 3 in brain region SN. In brain regions CC and CE, caspase 3 shows a significant (P < 0.05) increase in all the groups studied compared to control. In all the regions of the brain, D3 and D5 show a significant (P < 0.05) decrease in caspase 3 compared to the group treated with LPS. In all the brain regions, caspase 7 shows a significant (P < 0.05) increase in all the groups treated with LPS compared to control in all regions of brain. The group treated with LPS, D3, and D5 show a significant (P < 0.05) decrease in caspase 7 compared to the group treated with LPS. In all the brain regions, caspase 9
shows a significant ($P < 0.05$) increase in the groups LPS, D3, and D5 compared to control. In all the regions of brain, D3 and D5 show a significant ($P < 0.05$) decrease in caspase 9 compared to the group treated with LPS (Figure 11).
NRF2 significantly \((P < 0.05)\) increases in the LPS-treated group when compared with control in all the three regions of the brain (SN, CC, and CE). NRF2 decreases significantly \((P < 0.05)\) in both D3 and D5 groups when compared with LPS-treated ones in all the brain regions. Keap1 significantly \((P < 0.05)\) decreases in the LPS-treated group when compared with control in all the three regions of the brain. Keap1 increases significantly \((P < 0.05)\) in D3 and D5 groups when compared with LPS-treated ones in all the brain regions (Figure 12).

4. DISCUSSION

Studies revealed that the mean sizes of GNPs were 27 ± 4.3 nm by the AFM study and 20 ± 2.7 nm by TEM and the diameter of GNPs measured by DLS was reported to be approximately 37 nm.\(^{27}\) Interestingly, the presence of minus surface charge suggests the sodium citrate capping on the particle surface layer. We have targeted for small-sized FAuNP particle preparation, for making it further suitable in intestinal microvilli uptake during oral delivery and also for uptake in ex vivo cell culture studies. The FTIR spectrum of folate revealed various characteristic natures at frequencies 1606.37, 1694.51, and 1484.76 cm\(^{-1}\) possibly due to N–H bending of the CONH group, C=O amide stretching of the specific carboxyl group, and phenyl ring band, respectively. For citrate-reduced AuNPs, bands at 3453.62, 1272.50/1386.10, 621.60, and 2950 cm\(^{-1}\) represented OH bond stretching, carboxylic acid/C=O stretching of the carbonyl group connecting to Au, Au−O bond stretching, and C−H bond stretching, respectively.\(^{28}\) Finally, for FAuNPs, band frequencies at 3387.44 and 712.76 cm\(^{-1}\) represent OH bond stretching and Au−O bond stretching, respectively. Interestingly, intensities of the carbon-yl bands at 1606 and 1694 cm\(^{-1}\) in free folate were reduced in the case of FAuNPs. The pattern of vibrational stretching for the phenyl ring band at 1484 cm\(^{-1}\) in free folate was changed in the case of folate GNPs.

To overcome acidic environment of stomach and speedy metabolism in jejunum and ileum, nanoparticle-mediated drug delivery is always a better alternative. For slow and sustained drug release analysis, the \textit{in vitro} dialysis study of the FAuNP complex was carried out at acidic pH 3.0 and neutral pH 7.4 (Figure 3b). It generally takes around 2 h for FAuNP to go to the region of intestinal mucosal microvilli and thereby gets absorbed into the blood circulatory system to be transported to various organs. Interestingly, in the stomach, it only passes through before getting resided in the microvilli region (where it stayed for some time). Hence, when this drug complex was passing through acidic stomach (say around 30 min−1 h), the release was hardly 16–23%, whereas for neutral intestine (around 2 h), the release was only 20.6%. Therefore, this shows that the release profile of folate from FAuNP is totally pH-dependent and thereby establishes the slow, enhanced permeation retention effects of the particles.

The cell viability study of the gold nanocomplex on Hek293 and WI38 cells at doses varying from 0 to 50 μg/mL was performed for the incubation period of 3 h.\(^{29}\) Gold particles did not generate toxicological effects on HeLa and MCF7 cell line cultures after 4 h and 1 day incubation.\(^{29}\) The HEK 293 FR α negative (FRα−) cell line shows no uptake of free folate.
The pharmacokinetic study revealed that GNPs gradually could enter the circulatory system through absorption in the digestive system.30 However, fortunately, the body has several strategies to process and eliminate these entities through liver. No significant changes in mean body weight, food ingestion, and behavioral alterations were noted in nanoparticle-treated fish with respect to the NC group. The present exploration was also intended to demonstrate the chronotoxic effects of blank nanoparticles (AuNPs) and folate GNPs (FAuNPs) using quite a few critical markers like blood glucose, hemoglobin, cholesterol, triglyceride, ALP, and SGPT. Moreover, we observed no significant differences in glucose level, lipid profile (cholesterol and triglyceride), ALP, and SGPT among the groups, which indicated no notable hepatic toxicity of the vitamin-tagged metallic nanoparticle. Release of ALP and SGPT from the liver tissue into the blood stream characteristically signifies hepatocellular toxicity and damaged liver. However, the levels of all these factors in treated groups when compared to the NC group are found to be statistically insignificant. No notable differences were observed in the histological assessment of liver among different treatment groups. Almost ideal central vein, healthy sinusoids, and distinct nuclei were observed, demonstrating no perceptible hepatocellular toxicity.

Amplification in lipid peroxidation can be correlated to MDA production, which is one of the imperative indicators of accumulation of reactive oxygen species and oxidative damage.10 The increased MDA level in the LPS-treated group signifies superoxide radical generation and augmented oxidative load in the brain tissue. GNP treatment illustrated a drop in oxidative damage in brain, which results in lessening the MDA production. Amplification in the XO level indicates disparity between ROS accumulation and levels of endogenous antioxidants.31 XO activates xanthine dehydrogenase (XDH) through proteolysis to produce ROS during chronic oxidative damage and inflammation-induced brain damage. XDH mainly functions to generate hypoxanthine and xanthine to produce ROS under normoxic conditions. XO further promotes ROS production to persuade brain damage under acute and chronic hypoxic conditions. Therefore, XO plays a vital role in determining ROS production and protecting mitochondria from oxidative damage in the stressed brain. Nanoparticle-treated groups slightly reinstate the steadiness between ROS and antioxidants, thereby diminishing the XO level. Imbalance in the GSH system due to LPS treatment indicates a surplus of free radicals in the brain tissue, ensuing imbalance in GSH/
GSSG ratio. The property of nanoparticles predominantly to scavenge ROS was apparent from the results, as a sharp decrease in the levels of both enzymatic (SOD and CAT) and nonenzymatic antioxidants in the nanoparticle-treated groups was detected when compared to LPS ones. Such variation in brain antioxidants in response to the nanoparticle confirms the enhancement of the brain oxidative status against LPS-induced inflammation and oxidative damage.

Throughout the vertebrate system, instigation of oxidative stress response is synchronized by another influential and decisive transcription factor NRF2.3 Under normal cellular conditions, NRF2 is apprehended in latent condition in the cytoplasm by Keap1 protein. Keap1 negatively regulates NRF2 movement through interactions with a set of protein factors that employ ubiquitin ligase to accomplish the proteasomal degradation of NRF2.32 This explicit Keap1-dependant regulation of NRF2 instantaneously boosts the basal level of NRF2 in the nucleus and amplifies several NRF2-dependent gene expressions. In the current study, a sharp elevation in the nuclear level of NRF2 was observed in the brain exposed to LPS. However, a significant drop was noted in Keap1 expression in the LPS-treated group. In response to FAuNP treatment, Keap1 expression was noted to amplify, whereas a sharp reduction was recorded in NRF2 expression. Extreme oxidative stress and free radical amassing lead to the commencement of multiple transducers (kinases), which could phosphorylate both Keap1 and NRF2. These radicals may also directly attack the sulfhydryl-rich Keap1 protein that eventually may lead to conformational change in Keap1. Therefore, these string of events during the preliminary stage of oxidative stress dislocate the Keap1-NRF2 complex and stimulate the translocation of active NRF2 to the nucleus.33 In response to intracellular oxidative stress, NRF2 plays a decisive role as a regulator of the intracellular redox status. In the nucleus, NRF2 associates with a few small proteins, attaches directly to antioxidant response elements (AREs) of DNA, and commences the transcription of antioxidant genes, as we noted here in the brain tissue. Through interaction with the ARE, NRF2 neutralizes excessive ROS by activating the expression of certain genes associated with antioxidant factors. NRF2 is stimulated by oxidants in the LPS-infected damaged brain through the modification of critical cysteine thiols of Keap1 and/or phosphorylation of NRF2 protein. Consequently, enhanced expression of different crucial antioxidant enzymes is achieved from releasing NRF2 from Keap1 and translocating to the nucleus following nanoparticle administration to the damaged brain.

Neurotoxins such as LPS generally hinder nerve function due to excess amassing and surplus of ACh due to inhibition of the AChE enzyme.34 It is responsible for both neuronal and excitatory transmissions, which correlate with the activity of Na+−K+ pump. Decline in this enzyme in the LPS-treated groups hampers the ionic environment and Na+−K+ pump in fish brain. Our data indicate that LPS persuades caspase-dependent apoptosis in different regions of fish brain. Activation of the caspases ensures that the cellular components
Figure 11. Profile of (a) Caspase 3, (b) Caspase 7, and (c) Caspase 9 expressed in RDV (relative densitometric value) in different fish groups [1-control, 2-control + FAuNP, 3-LPS, 4-LPS + FAuNP(D3), 5-LPS + FAuNP(D5)] (data = mean ± standard error, n = 18). Different superscript alphabets denote significant differences (P < 0.05) in mean values between different treatment groups. Notes: three different brain segments taken into consideration for the experiments are SN, CC, and CE. Treatment groups: —control: no exposure, control + FAuNP: only FAuNP exposure, LPS: LPS exposure, LPS + FAuNP (D3): FAuNP exposure for 3 days after LPS exposure, and LPS + FAuNP (D5): FAuNP exposure for 5 days after LPS exposure.

Figure 12. Profile of (a) Nrf2 and (b) Keap 1 expressed in RDV (relative densitometric value) in different fish groups [1-control, 2-control + FAuNP, 3-LPS, 4-LPS + FAuNP(D3), 5-LPS + FAuNP(D5)] (data = mean ± standard error, n = 18). Different superscript alphabets denote significant differences (P < 0.05) in mean values between different treatment groups. Notes: three different brain segments taken into consideration for the experiments are SN, CC, and CE. Treatment groups: —control: no exposure, control + FAuNP: only FAuNP exposure, LPS: LPS exposure, LPS + FAuNP (D3): FAuNP exposure for 3 days after LPS exposure, and LPS + FAuNP (D5): FAuNP exposure for 5 days after LPS exposure.
are degraded in a gradual but controlled manner, carrying out cell death.33 Current results support the previous hypothesis that acute inflammation and brain injury could trigger apoptotic pathways in the cerebral and dorsal cortex region. Because apoptotic signaling is fundamental to many of the neurodegenerative diseases, targeting their reticence and fissure of this cascade mechanism is regarded as one of the potential therapies to treat such diseases.

The outcome of the present research showed that a well-characterized metallic nanoparticle is toxicologically safe and does not generate any harm to zebrafish. This will certainly have a benefit over some other conventional nanoparticles against brain inflammation and subsequent neurodegeneration. High physical stability and uptake of this nanoparticle is an additional benefit of FAuNPs. The study effectively illuminates the critical role of the NRF2-Keap1 factor to modulate and influence the expression of antioxidant and specific brain enzymes in LPS-infected fish. The current model could lead to identifying and comprehending the underlying mechanisms of how different proteins of the neurological system respond during inflammation-induced brain injury. This information will be valuable for the further development of drug-targeted strategies for delivery of FAuNPs as a successful therapeutic agent. The potential use of FAuNPs as neuroactive drugs is expected to improve the pharmacokinetic profile and, therefore, could be used exclusively for future brain research. It is extremely significant to note that the most efficient nanoparticle formulations for brain delivery still accumulate significantly in other regions of the body before being eliminated. Thus, it is important to design suitable nanoformulations that after reaching the brain are remotely triggered to release the drug instead of doing so in other tissues of the body. Future developments of nanoformulations will facilitate the clinical translation of the nanoparticles in the area of regenerative medicine. The development of such nanoparticles could also target specific brain cells during neurodegenerative disorders.

**AUTHOR INFORMATION**

**Corresponding Author**

Madhusudan Das — Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, India; orcid.org/0000-0002-9051-3082; Email: madhuzoo@yahoo.com

**Authors**

Sasanta Sadhukhan — Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, India

Mahammed Moniruzzaman — Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, India

Subhajit Maity — Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, India

Sudakshina Ghosh — Vidyanagar College for Women, Kolkata, West Bengal 700006, India

Arup Kumar Pattanayak — Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, India

Suman Bhushan Chakraborty — Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, India

Biswanath Maity — Translational Cell Biology Unit, Centre of Biomedical Research, Lucknow 226014, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00415

**Author Contributions**

S.S., M.M., and S.M. have contributed equally for the paper and are joint 1st authors.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We would like to acknowledge the West Bengal Science & Technology & Biotechnology (73 (sanc)-BT/P/BDget/RD-05/2017 dt.27.03.2018) for financial support.

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