

Research Article

Control of *In Vivo* Transport and Toxicity of Nanoparticles by Tea Melanin

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Nanoparticles are unfamiliar to researchers in toxicology. Toxicity may be generated simply due to the reduction in size. Compounds that prevent or cure toxic materials may not work on nanoparticles. Furthermore, as there are more and more applications of nanoparticles in drug delivery and *in vivo* imaging, controlling the transport and toxicity will be primary concerns for medical application of nanoparticles. Gold nanoparticles (GNPs) if injected intraperitoneally into mice can enter hippocampus and induce cognitive impairment. GNPs caused a global imbalance of monoamine levels, specifically affecting the dopaminergic and serotonergic neurons. Pretreatment of tea melanin significantly prevented the deposition of GNPs in mouse brains, especially in the hippocampus. Pretreatment of melanin completely alleviated GNP-induced impairment of cognition. Pre-administration of melanin stably maintained monoamines at normal profiles. Melanin completely prevented the invasion of GNPs into the Cornu Ammonis region of the hippocampus shown by coherent anti-Stoke Raman scattering microscopy. Here we show that the administration of tea melanin prevented the accumulation of Au in brain, the imbalance of monoamines, and the impairment of cognition in mice. The current study provides a therapeutic approach to toxicity of nanoparticles and a novel strategy to control the transport of GNP in mouse brain.

1. Introduction

Nanoparticles provide a novel platform for target-specific delivery of therapeutic agents [1–3]. Gold nanoparticles (GNPs) have recently been developed as an attractive candidate for use as carriers in drug and gene delivery, particularly because the gold core is essentially inert and nontoxic *in vitro* [4–7]. GNPs have been developed as drug carriers in pharmaceutical studies. This is largely due to the apparent benefits in targeting and medical image enhancement. Multiple conjugations can be achieved through the tight binding of sulfhydryl groups. In addition, GNPs are capable of passing both the blood-brain barrier and the blood-retinal barrier

[8]. However, neurotoxicity of GNPs has been reported to impair cognition in mice. In order to enhance the use of GNPs as drug carriers, the *in vivo* toxicity of nanoparticles must be minimized.

In general, GNPs exhibit very low cytotoxicity [9–16]. The cellular uptake of GNPs likely occurs through endocytosis, and is greatest for 50 nm particles [17]. The rare exception is that GNPs under 2 nm in diameter are toxic to many cell lines [18]. Furthermore, GNP uptake has been associated with damage to the cytoskeleton and cell adhesion [19].

GNPs show minor *in vivo* toxicity [20]. The tissue distribution of GNPs in rats and mice has been examined by inductively coupled plasma mass spectrometry (ICP-MS)

[21, 22]. GNPs 10 nm in diameter were present in the liver, spleen, kidney, testis, thymus, heart, lung, and brain, whereas GNPs larger than 50 nm were largely detected in the blood, liver, and spleen. GNPs ranging from 10 to 50 nm were found in the brain. In zebrafish embryos, silver nanoparticles produced almost 100% mortality, whereas GNPs induced minimal mortality at the same time point [23]. Previously, we have shown that GNPs can pass through the blood-retinal barrier and impair cognition in mice. To make better use of GNPs as a drug carrier to target the brain, the neurotoxicity must be eliminated.

We previously reported a polyphenolic antioxidant, of melanin nature, found in the black tea *Camellia sinensis* [24]. *Camellia sinensis* tea melanin comprises the high molecular weight portion of tea polyphenols [25]. Tea melanin has demonstrated a wide range of biochemical and pharmacological activities in animals including antioxidant activity, free radical scavenging, and immunomodulation [26–28]. Melanin also has revealed unexpected protective activity against various toxic substances, such as, benzidine, hydrazine, snake venoms, cisplatin, and acetaminophen [29–32].

In addition to antioxidant properties, one of the most significant properties of melanin is its chelating capability [33]. Melanin is capable of chelating heavy metals. In particular, melanin complexes with gadolinium (Gd) for use in Magnetic Resonance Imaging (MRI), thereby alleviating the *in vivo* toxicity of Gd [27]. In addition, melanin possesses paramagnetic properties due to a high concentration of free radicals conjugated to its polymeric matrix. The chelating and paramagnetic properties render melanin a promising candidate to scavenge GNPs *in vivo*.

2. Materials and Methods

2.1. Materials. HAuCl₄, sodium citrate, NaBH₄, HCl, HNO₃, H₂SO₄, H₂O₂, scopolamine, and other chemicals of analytical grade were purchased from Sigma-Aldrich and Fisher (United States). H₂O was obtained at >18 MΩ from a Milli-Q water purification system. Fully fermented black tea was purchased from local retail shops (Miaoli, Taiwan).

2.2. Animal Treatment. Animal treatments were performed following “The Guidelines for the Care and Use of Experimental Animals” of National Chiao Tung University, Taiwan. Four-week-old male BALB/c mice were housed at 22 ± 2°C with a 12 h light/dark cycle and were fed standard rodent chow and water *ad libitum*. Mice were randomly assigned to experimental or control groups consisting of 6–8 mice. The control group did not receive any treatment, and the experimental groups were as follows: a melanin-treated group, a 17 nm GNP-treated group, a 37 nm GNP-treated group, a melanin pretreated-plus 17 nm GNP-treated group, and a melanin pretreated-plus 37 nm GNP-treated group. GNPs were administered in a single dose intraperitoneally. For the passive avoidance test, an additional positive control that received scopolamine (1 mg/kg i.p.) was incorporated.

2.3. Preparation of Gold Nanoparticles. GNPs, with diameters of 17 and 37 nm, were synthesized as previously reported [34, 35]. The seed colloids were prepared by adding 1 mL of 0.25 mM HAuCl₄ to 90 mL of H₂O and stirring for 1 min at 25°C. A 2 mL volume of 38.8 mM sodium citrate was stirred into the solution for 1 min, and then 0.6 mL of freshly prepared 0.1 M NaBH₄ in 38.8 mM sodium citrate was added. Different diameters of GNPs ranging from 3 to 100 nm were generated by altering the volume of added seed colloid. The solution was stirred for an additional 5–10 min at 0–4°C. Reaction temperatures and times were adjusted to obtain larger GNPs. All synthesized GNPs were characterized by UV absorbance. The size of the synthesized GNPs was verified by electron microscopy and atomic force microscopy. GNPs were dialyzed against phosphate buffered saline (pH 7.4) before injection into the animals.

2.4. Extraction, Fractionation, and Characterization of Tea Melanin. Isolation of tea melanin was performed according to a previous report [25]. Monomeric polyphenols were removed by treating the tea leaves with boiling water at a ratio of 1:10 (m/v) for 10 min followed by filtration. The obtained solid matter was immersed in 40°C water at a 1:10 (solid/liquid) ratio, and the pH was adjusted to 10.5 with 10% NH₄OH. The extraction time was reduced to 12 h to avoid excessive oxidation of melanin. The extract was filtered and acidified to pH 2.5 using 2 N HCl and then centrifuged at 15,000 g for 15 min to form a pellet. Acid hydrolysis of the pellet was employed to remove carbohydrates and proteins. Organic solvents (chloroform, ethyl acetate, and ethanol) were used to remove lipids and related compounds. In addition, melanin was reduced by treatment with Ti⁺³. The reduced melanin was dialyzed against Milli-Q water to remove Ti⁺³. Finally, the reduced sample was suspended in distilled water, and 0.1 M NaOH was added drop wise to dissolve the melanin and to adjust the pH to 7.0. The solution was filtered through a Nalgene 0.45 μm filter. Melanin was fractionated on a Sephadex G-75 column (1.6 × 40 cm) at a 0.5 mL/min flow rate using 50 mM phosphate buffer (pH 7.5). Fractions were monitored by ultraviolet (UV) absorbance at 280 nm. All operations were conducted under N₂. The physical and chemical characteristics of melanin were examined as described. UV absorption spectra were recorded on a JASCO V-530 UV-Visible Spectrophotometer. Infrared (IR) spectra were recorded on a Perkin Elmer spectrometer (Model 1600 FT). The purity of melanin was examined by thin layer chromatography (TLC) using silica gel as the stationary phase and chloroform/ethyl acetate/formic acid (6:4:1) as the mobile phase. Melanin was retained at the origin in TLC separation and did not produce any additional signals on the chromatogram. Comparisons with TLC separation of caffeine, catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and theaflavin assured that the melanin preparation was free from these components.

2.5. Inductively Coupled Plasma Mass Spectrometry (ICP-MS). For total element determinations, standard solutions were

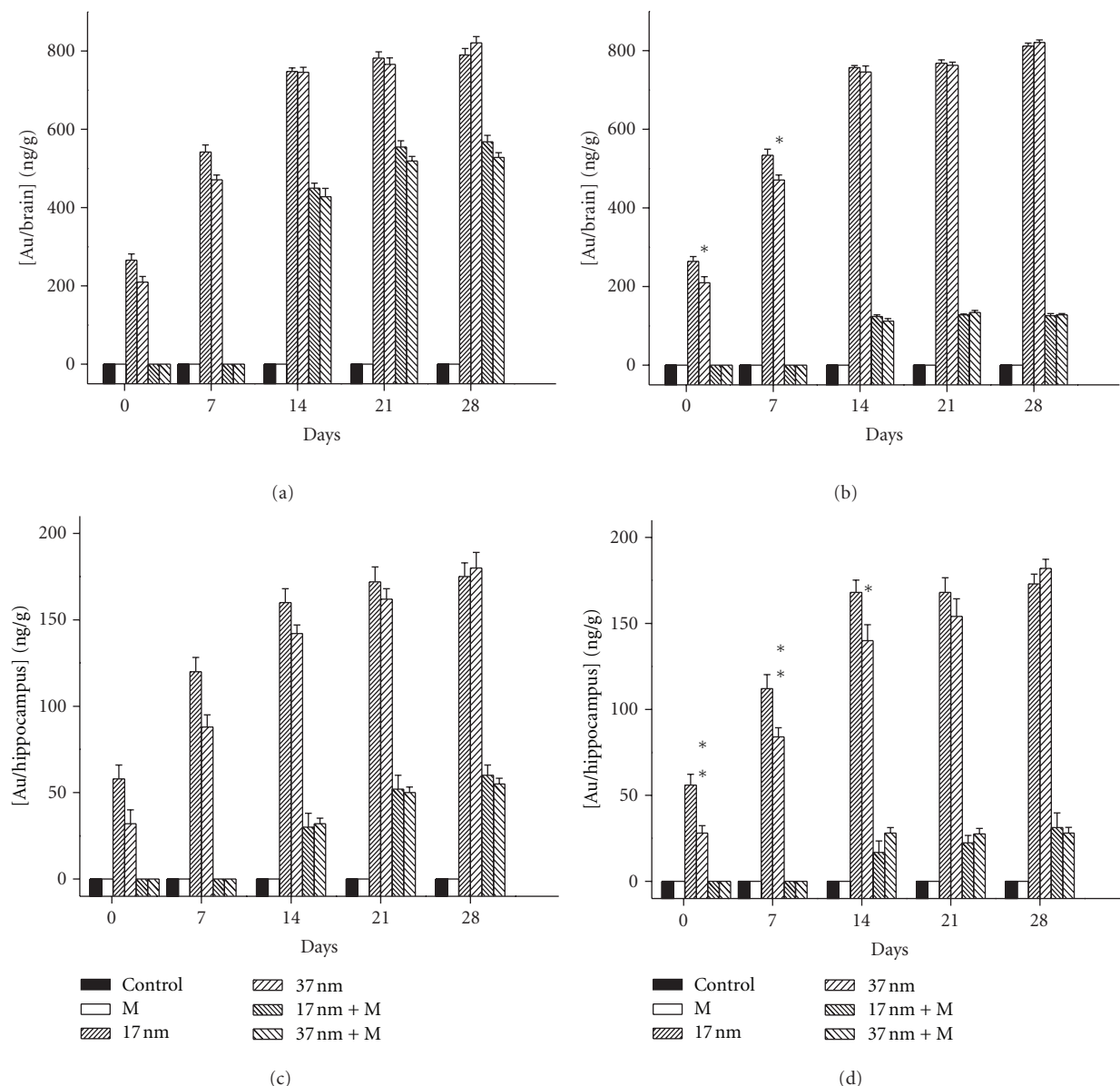


FIGURE 1: The effect of tea melanin on the accumulation of GNPs in the brain and hippocampus. Mice were divided into 6 groups: a control group receiving PBS; a melanin group receiving 0.3 or 0.5 mg/kg tea melanin; a 17 nm group receiving 10 mg/kg of 17 nm GNP; a 37 nm group receiving 10 mg/kg of 37 nm GNP; a 17 nm + M group receiving 10 mg/kg of 17 nm GNP and pretreated with 0.3 or 0.5 mg/kg melanin; a 37 nm + M group receiving 10 mg/kg of 37 nm GNP and pretreated with 0.3 or 0.5 mg/kg melanin. Each group included 30 mice. Six mice from each group were sacrificed on day 1, 7, 14, 21, and 28. The brain was carefully dissected and the hippocampus was separated. ICP-MS was performed to obtain the content of Au. (a) Deposition of Au in the hippocampus for mice receiving 0.3 mg/kg melanin. (b) Deposition of Au in the brain for mice receiving 0.3 mg/kg melanin. (c) Deposition of Au in the hippocampus for mice receiving 0.5 mg/kg melanin. (d) Deposition of Au in the brain for mice receiving 0.5 mg/kg melanin.

prepared by dilution of a multielement standard (1,000 mg/L in 1 M HNO_3) obtained from Merck (Darmstadt, Germany). Nitric acid (65%), hydrochloric acid (37%), perchloric acid (70%), and hydrogen peroxide (30%) of Suprapur grade (Merck) were used to mineralize the samples. A size-exclusion column was connected to the ICP-MS apparatus. Brain section samples were homogenized in 25 mM tris(hydroxymethyl) aminomethane (Tris)-12.5 mM HCl

buffer solution at pH 8 and centrifuged at 13,000 rpm for 1 h. The supernatant was applied to the size-exclusion column of the HPLC system, which had been equilibrated with 25 mM Tris-12.5 mM HCl (containing 20 mM KCl) and eluted with the same buffer at a flow rate of 1 mL/min. The metal components of metal-binding proteins that were eluted from the HPLC system were detected by ICP-MS (Perkin Elmer, SCIEX ELAN 5000). The main instrumental operating

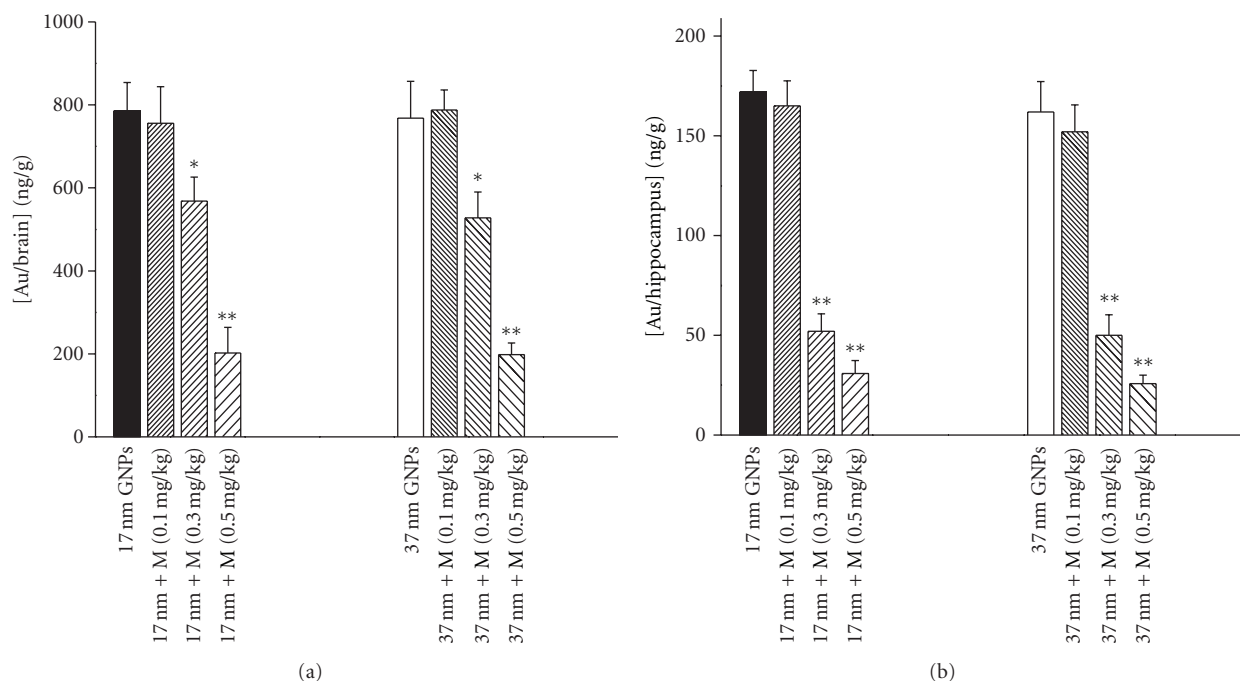


FIGURE 2: Dose-dependence for melanin-suppression of Au deposition in the brain and the hippocampus. Mice were pretreated with melanin at 0.1, 0.3, or 0.5 mg/kg and received 10 mg/kg 17 nm or 37 nm GNP. The accumulation of Au on day 21 was obtained. (a) Accumulation of 17 nm GNP (left) and 37 nm GNP (right) in the brain. (b) Accumulation of 17 nm GNP (left) and 37 nm GNP (right) in the hippocampus.

conditions were as follows: RF power 1900 W, carrier gas flow 0.8 L/min Ar, and makeup gas flow 0.19 L/min Ar. 197 Au was used as the internal standard.

2.6. Passive-Avoidance Test. The passive-avoidance apparatus consisted of two compartments with a steel-rod grid floor (36 parallel steel rods, 0.3 cm in diameter, set 1.5 cm apart). One of the compartments (48 × 20 × 30 cm) was equipped with a 20 W lamp located centrally at a height of 30 cm, and the other compartment was dark and of the same size, connected through a guillotine door (5 × 5 cm). The dark room was used during the experimental sessions that were conducted between 09:00 and 17:00 h. During the training trial, the guillotine door between the light and dark compartment was closed. When the mouse was placed in the light compartment with its back to the guillotine door, the door was opened, and the time until the mouse entered the dark compartment (step-through latency, STL) was measured with a stopwatch. After the mouse entered the dark compartment, the door was closed. An inescapable scrambled foot shock (1 mA for 2 s) was delivered through the grid floor. The mouse was removed from the dark compartment 5 s after the shock. Then, the mouse was put back into the home cage until the retention trial was carried out 24 hours later. The mouse was again placed in the light compartment, and as in the training trial, the guillotine door was opened and the STL was recorded and used as a measure of retention. If the mouse did not step through the door after 300 s, the experiment was terminated.

2.7. Analysis of Monoamine and Acetylcholine Concentrations in the Mouse Brain. Monoamine levels were determined as previously reported [36]. The mice were decapitated, and their brains were quickly removed. The brain samples were weighed and homogenized on ice using a polytron homogenizer (Kinematica, Lucern, Switzerland) at a maximum setting for 20 s in 10 volume equivalents of 0.2 M perchloric acid containing 100 mM Na₂-EDTA and 100 ng/mL isoproterenol. The homogenate was centrifuged at 15,000 g for 30 min. The pH was adjusted to approximately 3.0 using 1 M sodium acetate. After filtration (0.45 μm), the samples were separated using high-performance liquid chromatography (HPLC). Monoamines and their metabolites were separated using HPLC at 30°C on a reverse-phase analytical column (ODS-80, 4.6 mm i.d. × 15 cm) and detected with an electrochemical detector (Model ECD-100, Eikom Co., Kyoto, Japan). The column was eluted with 0.1 M sodium acetate-citric acid buffer (pH 3.5) containing 15% methanol, 200 mg/L sodium 1-octanesulfonate, and 5 mg/L Na₂-EDTA. The following monoamines and their metabolites were measured: norepinephrine (NE), 4-hydroxy-3-methoxyphenylglycol (MHPG), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT, serotonin), 5-hydroxyindoleacetic acid (5-HIAA), epinephrine (EPI), and homovanillic acid (HVA).

2.8. Ex Vivo Coherent Anti-Stoke Raman Scattering (Cars) Microscopy. Freshly excised hippocampi were dissected into thin slices, approximately 2 mm in thickness and immersed

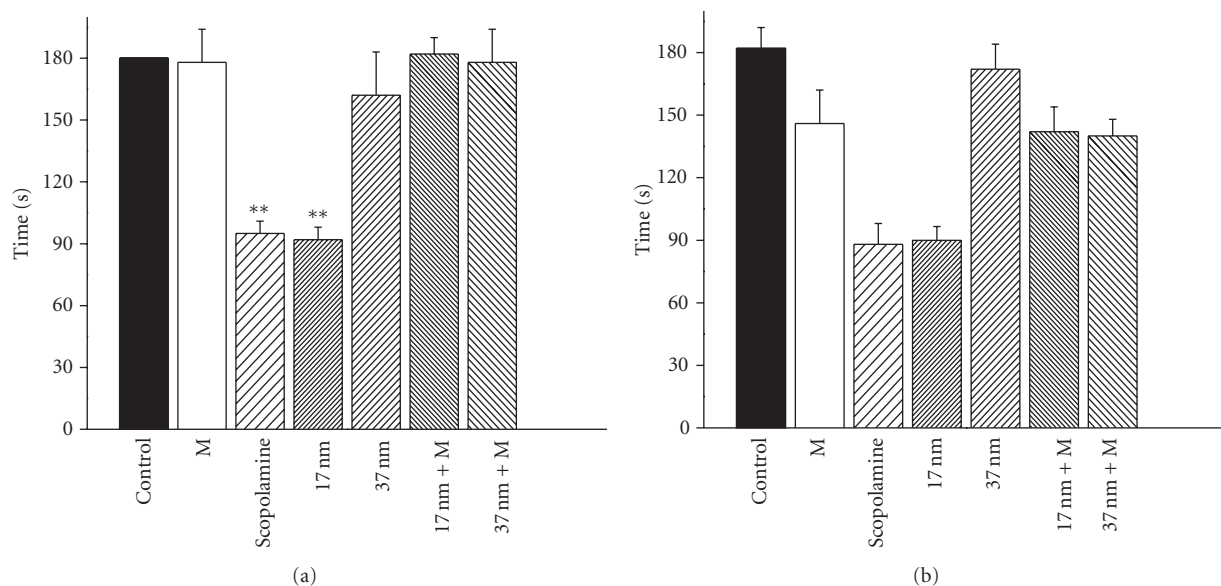


FIGURE 3: The effect of melanin pretreatment on GNP-induced cognition impairment. A passive avoidance test using light/dark rooms was performed. The latency time profiles on day 21 are shown for mice pretreated with 0.3 mg melanin/kg (a) and 0.5 mg melanin/kg (b). Each group of columns contains, in sequence, the averaged values from the control group (control), the melanin-treated only group (M), the scopolamine-treated group (scopolamine), the 17 nm GNP-treated group (17 nm), the 37 nm GNP-treated group (37 nm), the melanin pretreatment-plus 17 nm GNP-treated group (M + 17 nm), and the melanin pretreatment-plus 37 nm GNP-treated group (M + 37 nm). *represents $P < 0.05$ and **represents $P < 0.001$ from the student's t -test.

into a microchamber filled with PBS on a glass slide for examination. CARS microscopy was performed using a time constant of 3 ms, a scanning area of $300 \times 300 \mu\text{m}$, a step size of $1 \mu\text{m}$, a scanning size of 300×300 pixels, a scanning velocity of $1 \mu\text{m}/\text{ms}$, and a sampling rate of 80 kHz. Laser power was set at 30 mW for 870 nm and 40 mW for 1,064 nm. The wavelengths of the Pump and the Stokes lasers (Pump = 870 nm and Stokes = 1,064 nm) were tuned to match a Raman shift ($\sim 2100 \text{ cm}^{-1}$) that falls in the so-called "silent region" of the vibrational spectra of cells and tissues. As expected, the CARS images of the untreated hippocampus did not show appreciable contrast under the nonresonant condition. The CARS signals from GNP-treated specimens, however, were dramatically enhanced, that is, they appeared as scattered bright spots. The signal enhancement presumably resulted from strong scattering and large third-order polarizability of the GNPs [37–40].

2.9. Statistical Analyses. All data are presented as the mean \pm SD with a minimum of 6 mice per group. Concentrations of biogenic amines in mouse brains were analyzed using the unpaired Student's t -test. The criterion for statistical significance was set at $P < 0.05$ for all statistical evaluations.

3. Results and Discussion

3.1. Pretreatment of Melanin Reduced Au Accumulation in Brain and Hippocampus. Accumulation of Au in the brain was investigated (Figure 1). Mice were divided into 6 groups

as follows: a control group receiving PBS; a melanin group receiving 0.5 mg/kg tea melanin; 17 nm GNP group receiving 10 mg/kg of 17 nm GNPs; 37 nm group receiving 10 mg/kg of 37 nm GNPs; 17 nm + M group receiving 10 mg/kg of 17 nm GNPs and 0.3 or 0.5 mg/kg melanin 3 hours prior to GNP treatment; 37 nm + M group receiving 10 mg/kg 37 nm GNPs and receiving 0.3 or 0.5 mg/kg melanin 3 hours prior to GNP treatment. Each group included 30 mice. Six mice from each group were sacrificed on day 1, 7, 14, 21, and 28. The brains were carefully dissected and the hippocampi were separated. ICP-MS was performed to obtain measurements for the accumulation of Au. Without melanin pretreatment, GNPs accumulated rapidly in the brain and reached a plateau of approximately 770 ng/g brain on day 14 (Figure 1(a)). The accumulation of 17 nm GNPs was apparently faster than that of 37 nm GNPs in the first 14 days. However, both GNPs reached the same plateau and remained unchanged at later time points. Pretreatment of 0.3 mg/kg melanin effectively suppressed the accumulation of Au in brains during the first 2 weeks. However, an abrupt increase in Au accumulation was observed in brains at later time points (440 ng/g brain for 17 nm GNPs and 420 ng/g brain for 37 nm GNPs). The amount of accumulation for melanin pretreated groups was approximately 65% compared to the control group on day 14. This ratio was slowly increased to 75% at the end of the experiment. Administration of 0.5 mg/kg melanin significantly reduced the accumulation of GNPs in the brain from ~ 420 to 100 ng/g for 17 nm and 37 nm GNPs on day 14 (Figure 1(b)). The amount of Au was maintained at this level until the end of the experiment. Pretreatment of melanin at

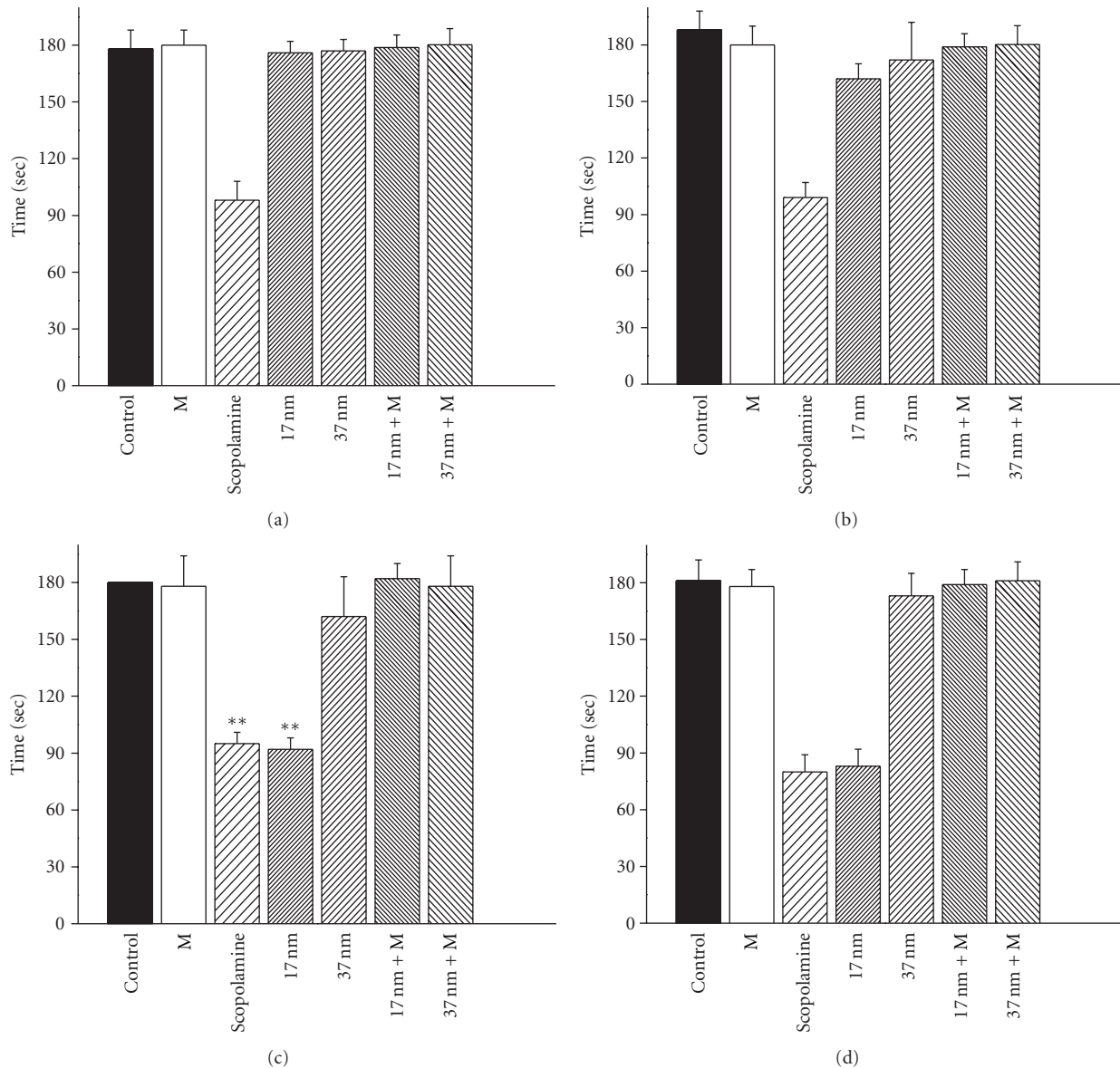


FIGURE 4: Time course of the passive-avoidance test performed on mice pretreated with melanin at 0.3 mg/kg. Mice were pretreated with melanin at 0.3 mg/kg followed by injection with 17 nm GNPs. The passive avoidance test was performed on day 7 (a), day 14 (b), day 21 (c), and day 28 (d).

0.5 mg/kg effectively suppressed the accumulation of GNPs in the brain.

In the hippocampus, the Au accumulation profile resembled that of the brain. The Au accumulation increased rapidly during the first two weeks and reached a plateau of ~160 ng/g of hippocampus on day 14. Pretreatment of melanin at 0.3 mg/kg decreased accumulated Au to a nondetectable level in two weeks (Figure 1(c)), but Au increased rapidly to 30 ng/g on day 14, reaching 60 ng/g on day 28. Pretreatment of melanin at 0.5 mg/kg decreased accumulated Au to a nondetectable level for the first two weeks (Figure 1(d)), but Au increased rapidly to 25 ng/g on day 14, remaining unchanged until day 28.

The suppression of Au accumulation by pretreatment of melanin was dosedependent (Figure 2). On day 21, pretreatment of 0.1 mg/kg melanin exhibited no influence on the suppression of Au accumulation in the brain (Figure 2(a)) and hippocampus (Figure 2(b)). Administration of 0.3 mg/kg melanin reduced Au accumulation by 30% for 17 nm GNPs and by 36% for 37 nm GNPs in the brain. Melanin at this dosage also reduced 71% Au accumulation for 17 nm and 37 nm GNPs in the hippocampus. Administration of 0.5 mg/kg melanin reduced 74% of Au accumulation for 17 nm GNPs and 37 nm GNPs in the brain. Melanin at this dosage also reduced Au accumulation by 82% for 17 nm GNPs and by 84% for 37 nm GNPs in the hippocampus.

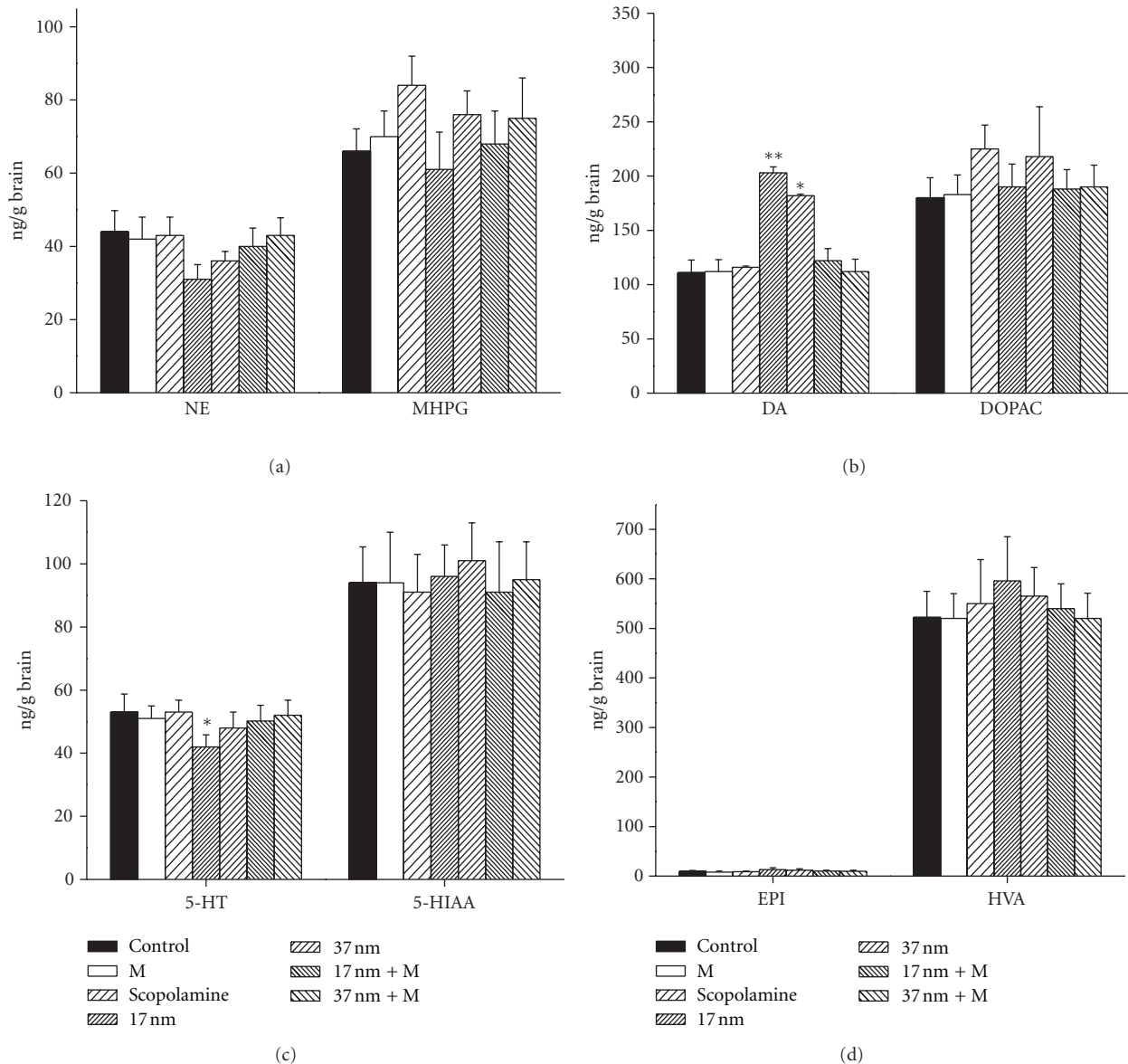


FIGURE 5: Melanin ameliorates the GNP-induced imbalance of neurotransmitter profiles. Immediately after the passive avoidance test, brain tissues were removed and the levels of monoamines were analyzed by HPLC. The levels of neurotransmitters and their metabolites are shown. (a) Norepinephrine (NE) and 4-hydroxy-3-methoxyphenylglycol (MHPG, metabolite). (b) Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC, metabolite). (c) 5-Hydroxytryptamine (5-HT, serotonin) and 5-hydroxyindoleacetic acid (5-HIAA, metabolite). (d) epinephrine (EPI) and homovanillic acid (HVA, metabolite). Each group of columns contains, in sequence, averaged values from the control group (control), the melanin-treated only group (M), the scopolamine-treated group (scopolamine), the 17 nm GNP-treated group (17 nm), the 37 nm GNP-treated group (37 nm), the melanin pretreatment-plus 17 nm GNP-treated group (M + 17 nm), and the melanin pretreatment-plus 37 nm GNP-treated group (M + 37 nm). *represents $P < 0.05$ and **represents $P < 0.001$ from the student's t -test.

3.2. Melanin Ameliorated GNP-Induced Cognition Impairment in Mice. GNP of 17 nm diameter impaired the cognition of mice, which was evident by the reduction in latency time measured for the passive avoidance test [41]. Pretreatment of melanin efficiently suppressed the accumulation of GNPs in mouse brains and hippocampi. However, it is not clear if the remaining GNPs would cause any physiological damage to the hippocampus. Mice were pretreated with 0.3 or 0.5 mg/kg melanin and injected with GNPs at

10 mg/kg. The passive-avoidance test was performed on day 21 (Figure 3). Scopolamine, serving as a positive control, shortened the latency time from 180 s to 90 s. Injection of 17 nm GNPs shortened the latency time from 180 s to 90 s. Pretreatment of melanin at a dosage of 0.3 mg/kg recovered the latency time to 180 s (Figure 3(a)). Melanin at 0.5 mg/kg also recovered the latency time to 140 s. Melanin is a mild sedative, and pretreatment with 0.5 mg/kg induced a minor neuronal disturbance, which was evident in the passive

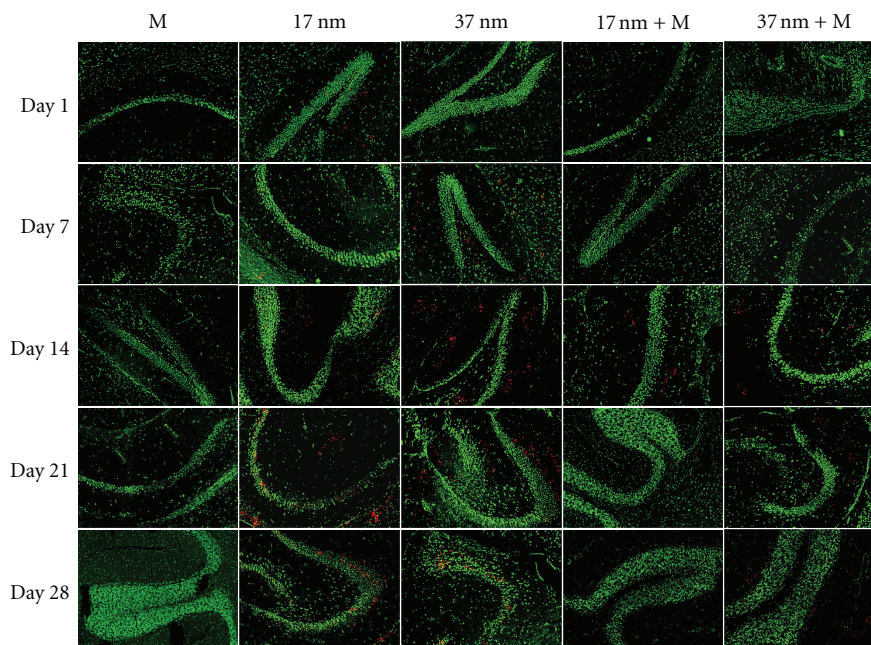


FIGURE 6: Time course examination by CARS microscopy of hippocampi isolated from mice in the melanin-treated only group (M), the 17 nm GNP-treated group (17 nm), the 37 nm GNP-treated group (37 nm), the melanin pretreatment-plus 17 nm GNP-treated group (M + 17 nm), and the melanin pretreatment-plus 37 nm GNP-treated group (M + 37 nm). The wavelengths of the Pump and Stokes lasers (Pump = 870 nm and Stokes = 1,064 nm) were tuned to match a Raman shift ($\sim 2,100 \text{ cm}^{-1}$) that fell in the so-called “silent region” of the vibrational spectra of cells and tissues. To better visualize the location of GNPs, the enhanced bright spots are colored red in the final images. The green fluorescence is the autofluorescence emitted from the cells of the CA region of the hippocampus.

avoidance test. A 20% reduction in latency was observed for the melanin-treated group (Figure 3(b); M, M + 17 nm, and M + 37 nm). Although melanin at 0.5 mg/kg reduced the accumulation of Au more efficiently than melanin at 0.3 mg/kg, the lower dose was selected for the remaining experiments due to the lower neuronal toxicity.

Mice were treated with melanin at 0.3 mg/kg prior to the administration of 17 nm or 37 nm GNPs. The passive avoidance test was performed on day 7, 14, 21, and 28 (Figure 4). On day 14, mice treated with 17 nm GNPs started to show cognition impairment, which was pronounced by day 21. Injection of 37 nm GNP, however, never showed symptoms of neurotoxicity throughout the course of experiment. Pretreatment of melanin was sufficient to suppress the neurotoxicity induced by 17 nm GNP injection.

3.3. Melanin Prevented the GNP-Induced Imbalance of Monoamines in Brain. A global imbalance of neurotransmitters in the brain has been associated with GNP treatment [41]. In particular, analysis for monoamines from brain tissues has indicated that treatment with GNPs elevates dopamine levels and decreases serotonin levels. Administration of GNPs affected the mouse dopaminergic and serotonergic neurons. Mice were pretreated with 0.3 mg melanin/kg and then injected with GNPs at 10 mg/kg. The levels of monoamines were measured (Figure 5). The levels of norepinephrine (NE) and epinephrine (EPI) were undisturbed. GNP injection elevated the level of dopamine (DA), which was alleviated

by the pretreatment of melanin. GNP also decreased the level of 5-hydroxytryptamine (5-HT, serotonin), which was also brought back to normal with melanin pretreatment (see Figure S1 in Supplementary Materials available online at doi:10.1155/2012/746960). The ameliorating effect of melanin pretreatment was validated by these monoamine profiles in the brain.

3.4. Melanin Retarded the Invasion of GNPs into the Hippocampus. The hippocampus is located in the medial temporal lobe of the brain, which belongs to the limbic system and plays major roles in short-term memory and spatial navigation. GNP injection impaired learning and memory in mice, and so the GNPs could have been transported through the blood, across the blood-brain barrier into the brain and into the hippocampus. Furthermore, 17 nm and 37 nm GNPs showed differential effects on the cognition impairment of mice, although the accumulation rate of both GNPs in the brain and hippocampus were strikingly similar. To localize GNPs, the freshly dissected hippocampi were examined using *ex vivo* CARS microscopy (Figure 6). GNPs are known to enhance the anti-Stokes Raman signal of nearby amino acids. Using the proper controls, enhancement visualized by CARS strongly indicated the presence of GNPs. Localized enhancement of the anti-Stokes Raman signal at an excitation wavelength of 817 nm was observed from the hippocampi removed from 17 nm and 37 nm GNP-treated mice. The Raman signal was completely absent from control

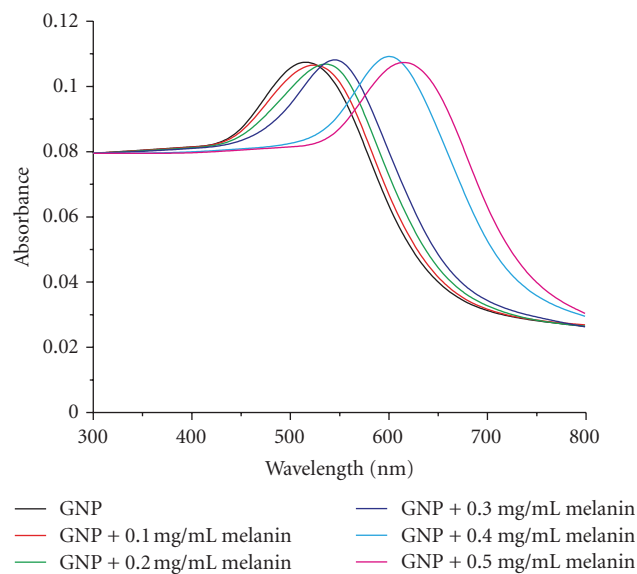


FIGURE 7: UV spectra for the titration of 17 nm GNP with melanin. Melanin is mixed with GNP at final concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL for melanin and 0.34 mM for GNP. UV absorbance of the binding reactions is shown. Red-shift of the absorbance peak indicates the increasing diameter of binding complex.

mouse tissues (mice pretreated with melanin). On day 21, the Raman signal of 17 nm GNPs was localized at the CA region of the hippocampus inside the cluster of neuronal cells, whereas 37 nm GNPs were less visible and scattered throughout the peripheral region. The distribution of 17 nm and 37 nm GNPs in the hippocampus suggests that the invasion of GNPs into the cluster of neuronal cells in the CA might have caused the learning impairment in the 17 nm GNP-treated mice. Accordingly, 37 nm GNPs were incapable of entering the neuronal cells, therefore causing only minimal deficits in learning and memory. The melanin-pretreated groups did not show scattered light throughout the whole study, which was consistent with our passive-avoidance test.

The *in vivo* toxicity of GNPs may very likely originate from the inability of the immune system to scavenge GNPs from organs. GNPs alone are biocompatible and stimulate a very minor immune response [42]. An overdose of GNPs, however, may cause a lethal effect in mice [20]. The lethality may be alleviated by the conjugation of immune-stimulating peptides to GNP.

The heavy metal chelating ability might play a role in the prevention of GNP nanotoxicity. Titration of 17 nm GNP by melanin was performed *in vitro* (Figure 7). In the titration experiment, melanin replaced citrate forming melanin-GNP complex. Melanin apparently has higher affinity than citrate to GNP binding. The melanin-GNP binding complex was stable in solution when heated up to 70°C for 10 min. Melanin is potentially capable of scavenging GNPs from blood stream.

Melanin is known to stimulate the immune system in mice [24]. The administration of melanin could enhance the ability of the mouse immune system to protect the invaded GNPs. However, it remains to be explored if this type of general immune response could protect the neuronal damage caused by GNPs' invasion into hippocampus.

4. Conclusion

Here we demonstrate the protective activity of tea melanin against GNP-induced neurotoxicity in mice. The administration of tea melanin was capable of retarding the accumulation of Au in the brain, recovering the imbalance of monoamines and preventing cognition impairment, thereby preventing GNP-induced neurotoxicity in mice.

Acknowledgments

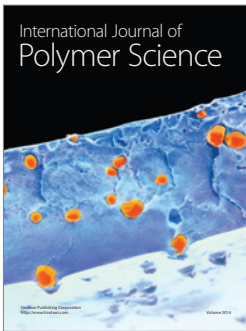
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