Article ID: NANO/364791/PAP
Title : Impairment of cognition in mice caused by the injection of gold nanoparticles
Dear Editors:
Thank you very much for your mail. We have re-examined our data and figures as suggested by the reviewer, carefully revised our manuscript, and made appropriate amendments in accordance to reviewer's comments/suggestions. Particularly, the revision was sent out and proofread by American Journal Experts. The certificate was attached with this response.
The following are the answers to reviewer's inquiry.
First referee's report
In this manuscript, the authors investigated the in vivo effects of gold nanoparticles on the learning and memory of mice. A mechanism of the size-dependent brain function impairment was also proposed based on the microscopic tests and the neurotransmitter measurement. The work does show some interesting data. Some useful information may be provided for the understanding of nanoparticle-induced neurotoxicity. However, in its current state, the presentation and the language of the manuscript cannot meet the desired standard for publication. Thus, I recommend reconsidering it after major revision.
Detailed comments are listed as below for the authors to improve their paper quality:
1. The title should be changed to "Size dependent impairment of cognition in mice……".
Answer: Thank you very much for the comments. The point is well taken. In the revision, we have changed the title to "Size-dependent impairment of cognition in mice caused by the injection of gold nanoparticles" as recommended.
Confidential: not for distribution. Submitted to IOP Publishing for peer review 4 October 2010

2. The abstract is too long. It should be reduced to one paragraph.
Answer: Thank you very much for the comments. The original abstract contains 363 words in 5 paragraphs. We re-organized the abstract and reduced wording. The revised abstract contains 246 words in 3 paragraphs. Because this study deals with multidisciplinary issue, we believe that proper introduction in the abstract could help general reader to comprehend the results more easily. However, the abstract in the present form can be combined into single paragraph if necessary.
3. There are too many redundant descriptions in this manuscript. For example, in the introduction part, the authors mentioned the advantages and applications

of GNP twice (Paragraph 1 and 3).
Answer: Thank you very much for the comments. The aforementioned sentence was eliminated in the revision. In addition, we also found several redundancies and modified in the revision.
(1) The third paragraph was reduced to two sentences in revision.
(2) Because passive avoidance test was described in detail in the Materials and Methods, the relative description in the Result section was simplified. (P14, L10-16 in the original manuscript was reduced to "Untreated mice exhibited a latency of 180 s." in the revision.)
4. The introduction should be organized and some new information should be added in. Besides the studies of GNP cytotoxicity, the authors should review the works on neurotoxicity of GNP. The sentence, which compared the mortality of silver NPwith GNP, should be deleted.
Answer: Thank you very much for the comments. We have added several new references as recommended by both reviewers in the introduction (Malugin, 2010; Qiu 2010; Rayavarapu 2010; Wang 2010; Cho 2009; Nel 2009; Zhang 2009; Dobrovolskaia 2007; Niidome 2006; Owens 2006). The sentence regarding comparison of silver and gold nanoparticles was summarized from description of the original reference. Due to the improper comparison, this particular reference was deleted in the revision.
5. SEM and TEM have been mentioned in the method part. The authors should provide the images as supporting information.
Answer: Thank you very much for the comments. TEM has been used to characterize GNPs. The images are included in the supporting information as recommended (Supporting information, Fig. 1S).

6. Most of the assays were conducted after 21 days. Why was the passive-avoidance test conducted on the 14th day, but not the 21st day? It is not acceptable to use the data on the 21st day to explain the behavior of mice on the 14th.
Answer: Thank you very much for the comments. We re-examined all our experimental records. Mice were treated for 21 days and characterization was performed. The "14th day" is apparently a mistake during the manuscript writing. We appreciate very much for the correction.
7. A simply drawing of the device used for the passive-avoidance test should be added into the figures.
Answer: Thank you very much for the comments. The point is well-taken. We have included picture displaying the complete layout of the passive avoidance apparatus in

the supporting information (Supporting information, Fig. 2S).
8. The authors did not mention the dose-dependent accumulation assay in the method part.
Answer: Thank you very much for the comments. The dose-dependent accumulation assay was incorporated into the “Animal treatment” section of Materials and Methods in the revision.
9. On page 13, line 8-12, how can the authors say that day 1 to day 14 is the first week?
Answer: Thank you very much for the comments. The point was well taken. The original paragraph was confusing and might be misleading. The experiment was meant to show the size-dependent and dose-dependent accumulation of GNPs in the brain.We decided to simplify this paragraph as shown in the revision (P14, L4-8).
10. The authors should explain the reasons to choose 3.7mg/kg for the passive-avoidance test. The latency should be expressed as mean+/- SD.
Answer: Thank you very much for the comments. The dose of 3.7 mg/kg was originally chosen to avoid lethal effect, but still showed minor symptoms. This is further explained in the revision (P15, L1-4).
All SD was incorporated in the latency in the revised version.
11. Scopolamine was applied to treat the positive control group. The neurotransmitter measurement shows that the effects of scopolamine are quite

different from those of the 17nm GNP. It may indicate that different pathways are involved in their neurotoxicity.
Answer: Thank you very much for the comments. The reviewer is exactly correct in this aspect. Scopolamine induced amnesia through alternative pathways different from GNPs. Dopamine levels and Serotonin levels in the brain were significantly altered by the injection of 17 nm and 37 nm GNPs. GNPs affected dopaminergic and serotonergic neurons. Further experiments dissecting pathways associated with GNP neurotoxicity is in progress.
12. The English write up should be improved. The manuscript should be proofread by a native speaker.
Answer: Thank you very much for the comments. The revision was sent out and proofread by American Journal Experts. The certificate was attached with this response.
Second referee's report
This manuscript described the possible damaging effects of gold nanoparticles (GNPs) on the cognition of mice by injecting 17 nm and 37 nm GNPs intraperitoneally into BALB/c mice. The dosage responses indicated that 17 nm

GNPs pass through the blood-brain barrier more rapidly than 37 nm GNPs, and that both GNPs reached their highest levels in the brain on day 21. In this case, this MS will be of interest to the readers. The MS is well organized.
It is highly recommended that the authors consider the following:
1) The Abstract part is too long and does not match the format.
Answer: Thank you very much for the comments. The original abstract contains 363 words in 5 paragraphs. We re-organized the abstract and reduced wording. The revised abstract contains 246 words in 3 paragraphs. Because this study deals with multidisciplinary issue, we believe that proper introduction in the abstract could help general reader to comprehend the results more easily. However, the abstract in the present form can be combined into single paragraph if necessary.
2) It is quite interesting that Au NPs can enter the brain through BBB. However, the author did not make proper explanation. On the other hand, what is the suface group on the Au NPs that may influence the uptake? When Au NPs

injected into mice, they may bind with protein and change the zeta potential quickly, the relationship between the adsorption protein and uptake efficiency are not clarified, however this topic is mentioned in the review of Nel et al. (A.E. Nel, L. et al, Understanding biophysicochemical interactions at the nano-bio interface, Nat. Mater. 8 (2009) 543-557, Qiu Y, Liu Y, et al. Surface chemistry and aspect ratio mediated cellular uptake of Au nanorods, Biomaterials, 31: 7606-7619.)
Answer: Thank you very much for the comments. The point was well-taken. We have learned so much during the revising process. We have incorporated the suggested references into extra paragraph of Discussion section in revision.
(P22, L4-12) Binding of citrate at the gold surface was dynamic. It is possible that surface modifications that could have replaced citrate on the GNPs could have occurred after injection. Proteins such as albumin, immunoglobulins, complement, fibrinogen, and apolipoproteins bind strongly to nanoparticles once the particles are in body fluids [42]. In particular, binding between complement and immunoglobulin (opsonization) promotes receptor mediated phagocytosis [43, 44]. Binding of plasma protein is important for determining the in vivo biodistribution of nanoparticles. This binding might explain how the injected GNPs passed through blood brain barrier and entered into hippocampus.
3) The author showed the fluctuation of monoamine and acetylcholine levels in the whole brain. And showed global distribution of 17 nm GNPs in the six areas of mouse brain. It would be better to show the monoamine and acetylcholine levels in the corresponding brain areas accordingly. In addition, the monoamine

levels in different area are quite different. I would recommend if they can analyze the monoamine levels in the individual area, like hippocampus, striatum, etc.

Answer: Thank you very much for the comments. The analysis of monoamine demonstrated possible neuronal systems that are affected by the injection of GNPs. We would be more than happy to analyze monoamine levels in the dissections of brain, analyze genetic expression, and also search for possible therapy to prevent this type of damage. The proposed experiments were currently in progress and will be submitted as separated manuscript.

4) It is not very clear how they separate the six brain area for distribution by ICP-MS. Usually, it is dissected by Cortex, hippocampus, striatum, olfactory bulb, and so on.

Answer: Thank you very much for the comments. We agree that the dissection of six brain area is not conventional. This is in part due to the small size of mouse brain and also our instrumental ability to perform such delicate operation. However, with different materials, such as rat brain, the proposed experiment will be available to us. The hippocampus, at least, will be analyzed in much detail in our next experiments. The six areas, however, could be indication of overall biodistribution of GNPs once getting in the brain.

5) Several reports below about the biodistribution and safety of gold nanomaterials including rods and spheres should be learned and cited carefully.

References:

1) Niidome T, Yamagata M, Okamoto Y, Akiyama Y, Takahashi H, Kawano T, Katayama Y, Niidome Y (2006) J Control Release 114:343-347

2) Wang L, Li YF, Zhou LJ, Liu Y, Meng L, Zhang K, Wu XC, Zhang LL, Li B, Chen CY. (2010) Anal. Bioanal. Chem. 396:1105-1114

3) Cho WS, Cho MJ, Jeong J, Choi M, Cho HY, Han BS, Kim SH, Kim HO, Lim YT, Chung BH, Jeong J (2009) Toxicol Appl Pharmacol 236: 16-24

4) Zhang GD, Yang Z, Lu W, Zhang R, Huang Q, Tian M, Li L, Liang D, Li C (2009) Biomaterials 30: 1928-1936

5) Qiu Y, Liu Y, et al. Surface chemistry and aspect ratio mediated cellular uptake of Au nanorods, Biomaterials, 31: 7606-7619

6) Arnida, Malugin A, Ghandehari H. Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres. J Appl. Toxicol. 2010, 30, 212

7) Rayavarapu RG, Petersen W, Hartsuiker L, Chin P, Janssen H, van Leeuwen FWB, et al. Nanotechnology 2010, 21, 145101

Answer: Thank you very much for the comments. We have incorporated the abovementioned references into separate part of Introduction. We believe that the Introduction, in its current form, contains wider and more updated scope than before. All authors appreciate comments from reviewers. They are constructive and to the point. We believe that the manuscript in the present form is much more comprehensive for general readers.
Thank you very much for all the comments.

Sincerely,
G. Steve Huang
Institute of Nanotechnology
National Chiao Tung University
gstevehuang@mail.nctu.edu.tw

1

Impairment of cognition in mice caused by the injection of gold nanoparticles

Yu-Shiun Chena, Yao-Ching Hungb, Li-Wei Linb, Ian Liauc, Meng-Yeng Honga, and G. Steve Huanga, *
a Institute of Nanotechnology, Department of Materials Science and Engineering, National Chiao Tung University, 1001 University Road, Hsinchu 300, Taiwan (R.O.C.)
bSection of Gynecologic Oncology, Department of Obstetrics and Gynecology, China Medical University and Hospital, 91 Hsueh Shih Rd., Taichung 404, Taiwan, (R.O.C.)
cDepartment of Applied Chemistry, National Chiao Tung University, 1001 University Road, Hsinchu 300, Taiwan (R.O.C.)
*Corresponding author: Institute of Nanotechnology, Department of Material Science and Engineering, National Chiao Tung University, Hsinchu, Taiwan, R.O.C. E-mail address: gstevehuang@mail.nctu.edu.tw, Fax: +886 3 5729912. Tel: +886-3-5131451

2

Abstract

This study explored the possible damaging effects of gold nanoparticles (GNPs) on the cognition of mice by injecting 17 nm and 37 nm GNPs intraperitoneally into BALB/c mice at doses from 0.5 to 14.6 mg/kg. ICP-MS was performed on brain tissue collected 1, 14, and 21 days after injection to obtain the concentration of GNPs. The dosage responses indicated that 17 nm GNPs pass through the blood-brain barrier more rapidly than 37 nm GNPs, and that both GNPs reached their highest levels in the

brain on day 21.
To assay for defects in short-term memory, a passive-avoidance test was performed on day 21 for mice injected with 3.7 mg/kg GNPs. Treatment with 17 nm GNPs decreased the latency time from 180 sec to 81 sec ($p < 0.01$), comparable to the effect of scopolamine treatment (92 sec), while 37 nm GNPs showed no significant effect.
Both 17 nm and 37 nm GNPs caused a global imbalance of monoamine levels. Analysis of brain tissues for monoamines indicated that treatment with GNPs elevated dopamine levels from 114.5 ng/g brain to 143.6 ng/g brain for the 17 nm GNPs ($p < 0.01$) and to 138.2 ng/g brain for the 37 nm GNPs ($p < 0.05$). Serotonin levels were significantly reduced after treatment with the 17 nm GNPs from 57.2 ng/g brain to 44.3 ng/g brain ($p < 0.05$). Administration of both GNPs affected the mouse


dopaminergic and serotonergic neurons.
The microscopic distribution of GNPs in the hippocampus was examined using coherent anti-Stoke Raman scattering (CARS) microscopy. The 17 nm GNPs were identified at the Cornu Ammonis (CA) region of the hippocampus and inside the cell-clustered area, while 37 nm GNPs were excluded from the CA region. Transmission electron microscopy (TEM) verified the location of the 17 nm GNPs in the cytoplasm of pyramidal cells, while the 37 nm GNPs were only found outside neuronal cells.
In summary, this study shows that the ability of GNPs to damage cognition in mice is size-dependent and is associated with their ability to invade the hippocampus. The dosage and duration of treatment should be taken into account if GNPs are to be applied as vehicles carrying therapeutic agents into brain.
Keywords: gold nanoparticles, nanotoxicity, hippocampus, mice, learning impairment, monoamines, transmission electron microscopy.


## 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 because they possess several unique chemical and physical properties for the transportation and delivery of pharmaceuticals [4-7]. One principal advantage of these carriers is that the gold core is essentially inert and non-toxic. However, the toxicity of these particles due to their decrease in size has yet to be carefully examined.

The cytotoxicity of GNPs has previously been examined [8, 9], and GNPs may or may not be toxic to cell lines depending on the GNP size and surface modifications and the cell type used [10-15]. The cellular uptake of GNPs probably occurs through endocytosis and is greatest for 50 nm particles [16]. Once particles enter cells, certain types of GNPs under 2 nm in diameter are toxic to many cell lines, while larger GNPs exhibit no toxic effects [17]. Although no apparent cytotoxicity has been found, GNP uptake has been associated with damage to the cytoskeleton and cell adhesion [18]. The tissue distribution of GNPs in rats and mice has been examined in vivo by inductively coupled plasma mass spectrometry (ICP-MS) [19, 20]. GNPs of 10 nm in diameter were present in the liver, spleen, kidney, testis, thymus, heart, lung, and brain, while GNPs larger than 50 nm were largely detected in the blood, liver, and



spleen. In particular, GNPs ranging from 10 to 50 nm were found in the brain. In zebrafish embryos, silver nanoparticles produced almost 100% mortality, while GNPs produced minimal mortality at the same time point [21]. GNPs can pass through the blood-retinal barrier; however, retinal toxicity was not observed [22].

GNPs have been developed as drug carriers in pharmaceutical studies. This is largely due to the apparent benefits in targeting. GNPs are capable of enhancing medical imaging. Multiple conjugations are achieved through the tight binding of sulfhydryl group. In particular, GNPs are capable of passing blood brain barrier and blood–retinal barrier [22]. It is important to investigate the physiological impact of naked GNPs once they get into the brain. This study will provide additional insight into the use of GNPs as carriers in drug delivery to brain.

## Materials and methods

### Materials

HAuCl4, sodium citrate, NaBH4, HCl, HNO3, H2SO4, H2O2, and other chemicals of analytical grade were purchased from Sigma-Aldrich and Fisher United States. H2O was obtained at >18 M from a Milli-Q water purification system.

### Preparation of gold nanoparticles



Gold nanoparticles (GNPs) with diameters of 17 and 37 nm were synthesized as previously reported [23, 24]. 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 volume of 2 mL 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 changing the volume

of seed colloid added. 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. The potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed GNPs was characterized by zeta potentials (Table 1). Zeta potentials of both GNPs fall between ±40-60 mV, indicating good stability of colloidal gold in the solution. GNPs were dialyzed against phosphate-buffered saline (pH 7.4) before injection into the animals to avoid the toxicity of the buffer, such as endotoxin.

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 four groups consisting of 8–10 mice, including a control group consisting of mice that did not receive any treatment, a positive control that received scopolamine (1 mg/kg i.p.), a 17 nm GNP-treated group, and a 37 nm GNP-treated group. GNPs were administered in a single dose intraperitoneally. A passive-avoidance test was performed on the 14th day after the administration of GNPs. Animals were sacrificed at the end of the experiment by cervical dislocation, after which the brain was isolated and weighed. Excised samples were washed with normal saline and stored at -70 ºC for further assays.

Passive-avoidance test [25]

The 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 x 20 x 30 cm) was equipped with a 20 W lamp located centrally at a height of 30 cm, and the other was a dark compartment of the same size, connected through a guillotine door (5 x 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 footshock (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 step-through latency was recorded and used as a measure of retention. If the mouse did not step through the door after 300 s, the experiment was ended.

Analysis of monoamine and acetylcholine concentrations in the mouse brain

Monoamine levels were determined as previously reported [25]. 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 vol. equiv. 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 um), the samples were separated using high

9

performance liquid chromatography (HPLC). Monoamines and their metabolites were separated using HPLC at 30 oC on a reverse-phase analytical column (ODS-80, 4.6 mm i.d. x 15 cm) and detected by an electrochemical detector (Model ECD-100, Eikom Co., Kypto, 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-hydroxytyramine (5-HT, serotonin), and 5-hydroxyindoleacetic acid (5-HIAA).

Acetylcholine (Ach) levels were determined as previously described [25]. The HPLC system (DSA-300, Eicom) consisted of a detector with a platinum electrode. A guard column and an enzyme column were placed before and after the analytical column (4.6 mm × 160 mm, Eicompak AC-GEL; Eicom), respectively. Isopropylhomocholine was added to the sampling tubes as an internal standard, and the mixture was analyzed using HPLC. The mobile phase was 0.1 M phosphate buffer (pH 8.3), and the flow rate was 0.6 mL/min. Ach levels in the sample were quantified using the internal standard method.

Statistical Analyses

10

All data are presented as mean ± SD with a minimum of six rats in each group.

Concentrations of biogenic amines and Ach in mouse brains were analyzed using the unpaired student t-test. The criterion for statistical significance was $p < 0.05$ for all statistical evaluations.

Inductively coupled plasma mass spectrometry (ICP-MS)

For the total elements determinations, standard solutions were prepared by dilution of a multi-element standard (1,000 mg/L in 1 M HNO3) 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 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. 197Au was used as the internal standard.

Ex vivo coherent anti-Stoke Raman scattering (CARS) microscopy

Freshly removed hippocampi were dissected into thin slices, approximately 2 mm in thickness, and immersed in a microchamber on a glass slide under PBS for examination. CARS microscopy was performed with a time constant of 3 ms, a scanning area of 300 x 300 μm, a step size of 1 μm, 300 x 300 pixels, a scanning velocity of 1 μm/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 (~ 2100 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 “control” did not show appreciable contrast under the non-resonant condition, whereas the CARS signals were dramatically enhanced, i.e., they appeared as scattered bright spots on the images taken from the GNP-treated specimens. The enhancement presumably resulted from strong scattering by the GNPs and the large third-order polarizability of the GNPs [26-29].

Transmission electron microscopy (TEM)

Small pieces of unfixed tissue were fixed in 2.5% glutaraldehyde with 0.05 M sodium cacodylate-buffered saline (pH 7.4) at room temperature for 2 h. The primary fixation was followed by three 20 min washes with 0.05 M sodium cacodylate-buffered saline (pH 7.4). The samples were then placed into a 1% OsO4 solution in the same buffer at room temperature for 1 h. OsO4 fixation was followed by three 20 min distilled-water washes and dehydration in acetone. The samples were transferred successively to 33% and 66% Spurr resin/acetone solutions, with a 30 min incubation in each solution. The samples were then transferred to 100% Spurr resin, first for 5 h and then in fresh resin overnight. The samples were section into 100 nm sections using an ultra microtome. The grids with ultrathin sections were post-stained with uranyl acetate for 30 min followed by lead for 3 min. After the post-staining procedure, a thin layer of carbon was evaporated onto the grid surfaces. Ultrathin-sectioned material was examined with a Jeol 1400 and a 3200 FS TEM.

Results and discussion

Dose-dependent biodistribution of GNPs in mouse brain

GNPs (17 and 37 nm in diameter) were synthesized according to published procedures [23, 24]. The synthesis was monitored by UV absorbance, and particle



size was examined by electron microscopy (17 ± 1.5 nm and 37 ± 2.1 nm). The potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed GNPs was characterized by zeta potentials (Table 1). Zeta potentials of both GNPs fall between ±40-60 mV, indicating good stability of colloidal gold in the solution. The purified GNPs were injected intraperitoneally into BALB/c mice at doses of 0.5 to 14.6 mg/kg. ICP-MS was performed on brains sampled at 1, 14, and 21 days after the injection to evaluate residual GNPs in the brain (Figure 1). GNPs were detected in brain samples 1 day after injection. The difference between GNP levels on day 14 vs. day 1 was greater than the difference between days 21 and day 14, indicating that GNPs accumulated rapidly in the first week and then the accumulation slowed down in the second week, although the GNP amounts continued to increase. For all dosages, the amount of 17 nm GNPs deposited in the brain was approximately 20% higher than that for 37 nm GNPs on days 1 and 14, while the levels were similar on day 21. It is likely that 17 nm GNPs passed through the blood-brain barrier more readily than 37 nm GNPs based on the faster initial accumulation; however, the GNP levels were comparable on day 21. When the dosage of either GNP was higher than 7.3 mg/kg, symptoms of toxicity were noted in mice from day 21. The treated animals showed fatigue, loss of appetite, change in fur color,

and weight loss. Starting from day 21, the mice showed a significantly camel-like



back and a crooked spine. This is consistent with previous results showing that high doses of GNPs induced multiple abnormalities in mice [30].

GNPs impair learning and memory in mice

To explore if the injected GNPs retard brain function in mice, particularly learning and memory, we examined the passive-avoidance performance of GNP-treated mice at the dose of 3.7 mg/kg. Previously, we have demonstrated lethal dose of GNPs (8 mg/kg/week, for 4 consecutive weeks). However, the current study employed lower doses which only caused minor symptoms in mice. Scopolamine induces amnesia and was applied as the positive control (Fig. 2A). The device consisted of two compartments with a steel-rod grid floor. One of the compartments was equipped with a light source, and the other was dark. In the training trial, the mouse was placed in the light compartment. An electric shock was delivered as a penalty to the mouse for entering the dark compartment. Twenty-four hours after the training trial, the mouse was placed at the same location, and the latency time was recorded. Untreated mice hesitate to enter the room where they received a shock, exhibiting a latency of 180 sec.

Scopolamine induces amnesia, resulting in an avoidance latency of 92 sec, a 50% reduction compared to the untreated control ($p < 0.01$). Although both GNPs caused weakness in mice, an insignificant reduction in latency was observed for the 37 nm



GNP-treated mice, while the 17 nm GNP-treated mice showed a latency of 81 sec in the passive-avoidance performance test ($p < 0.01$), comparable to the amnesia caused by scopolamine treatment. Apparently, 17 nm GNPs cause amnesia in mice, while 37 nm GNPs have no effect.

Dosage response to various concentrations of 17 nm GNP was performed. Passive-avoidance test was performed to mice injected with 17 nm GNPs at doses of 0,

0.4, 0.8, 1.9, 3.7, 7.3, and 14.6 mg/kg. The lowest concentration of GNPs with the significantly reduced latency time (138s) comparing to control group is 1.9 mg/kg ($p<0.05$). The concentrations below 1.9 mg/kg have no significant effects. Latency time at higher doses is dose-dependent and plateaus rapidly at 7.3 mg/kg.

The monoamine and acetylcholine concentration profiles in mouse brain were significantly affected by GNPs

Formation and consolidation of learning and memory are associated with the

activity of neurotransmitter systems such as the acetylcholinergic, norepinephrinergic, dopaminergic, and serotonergic neurons [31]. Most neurotransmitter systems can influence learning and memory in mice. GNP-treatment induced learning impairment, indicating that GNPs might cause an imbalance of neurotransmitters in the mouse brain (Fig. 3). Norepinephrine negatively regulates the learning and memory process

16

[32]. Administration of scopolamine, 17 nm GNPs, and 37 nm GNPs did not affect the levels of norepinephrine and its metabolite MHPG. Activation of the dopaminergic system also causes learning impairment [33, 34]. GNP treatment elevated levels of dopamine from 114.5 ng/g brain to 143.6 ng/g brain for the 17 nm GNPs ($p < 0.01$), and to 138.2 for the 37 nm GNPs ($p < 0.05$). Serotonin was significantly reduced from 57.2 ng/g brain to 44.3 ng/g brain ($p < 0.05$) upon treatment with 17 nm GNPs [35] . GNP-induced learning impairment correlated with an increase of dopamine and a decrease of serotonin in the mouse brain.

The macroscopic distribution of 17 nm and 37 nm GNPs in the brain were indistinguishable

The differential effects of 17 nm and 37 nm GNPs on the cognition of mice implied that the distribution of GNPs in the brain might be size-dependent. ICP-MS was used to detect the distribution of GNPs in the mouse brain. After 21 days of GNP injection at the dose of 3.7 mg/kg, mouse brains were dissected into six parts: the left and right frontal lobes, left and right medial temporal lobes, and left and right occipital lobes. Both 17 nm and 37 nm GNPs were detected in all parts of the brain at concentrations ranging from 496.2 to 559.5 ng/g brain, and the lowest concentration was found in the occipital lobes (Fig. 4). We were unable to differentiate the

17

macroscopic distributions of 17 nm and 37 nm GNPs in mouse brains. The ICP-MS results indicated that 17 and 37 nm GNPs were capable of passing through the blood-brain barrier and entering the mouse brain.

CARS microscopy differentiated the local distributions of 17 nm and 37 nm GNPs in the hippocampus

The hippocampus is located in the medial temporal lobe of the brain, belongs to the limbic system, and plays major roles in short-term memory and spatial navigation. Since GNP injection impaired learning and memory in mice, the GNPs could have been transported through the blood, across the blood-brain barrier into the brain, and into the hippocampus. To verify the presence of GNPs, the freshly dissected hippocampi were observed using ex vivo CARS microscopy (Fig. 5). GNPs are

known
to enhance the anti-Stoke Raman signal of nearby amino acids. With proper controls, the enhancement made possible by CARS strongly indicated the presence of GNPs. GNPs were also diffused ex vivo into brain tissues to verify the enhancement of the Raman signal. Localized enhancement of anti-Stoke 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 mouse tissues. The Raman signal of 17 nm GNPs was localized at the Cornu



Ammonis (CA) region of the hippocampus inside the cluster of neuronal cells, while 37 nm GNPs were scattered throughout the peripheral region. The distribution of 17 nm and 37 nm GNPs in the hippocampus suggest 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, while 37 nm GNPs were incapable of entering neuronal cells, therefore causing only minimal deficits in learning and memory.

TEM revealed that 17 nm GNPs were located in the cytoplasm of hippocampal neurons while 37 nm GNPs were not

TEM was performed to verify the cytoplasmic location of the 17 nm and 37 nm GNPs in the hippocampus 21 days after injection of GNPs (Fig. 6 and Fig. 7). A total of 72 TEM images were examined. We found that the 17 nm GNPs were located in the cytoplasm of pyramidal cells (Fig. 6A, 6B, and 6C). The Au composition was verified using energy dispersive X-ray spectroscopy (EDS; Fig. 6F), and the gold was also detected using HR-TEM (Fig. 6E). The 17 nm GNPs were found to be associated with dendrites (Fig. 6D). In particular, the 17 nm GNPs were surrounded by coated pit-like structures in the cytoplasm, leading us to suspect that the 17 nm GNPs entered the cells through endocytosis. However, no endocytosis-related structure was found for the 17 nm GNPs at the dendrites. This implies that these GNPs entered the



dendrites through free diffusion. These results suggest that alternative mechanisms for the cell uptake of GNPs occur together, as previously reported. Invasion of metallic particles such as GNPs into the neuronal cells and dendrites could seriously interfere with the electric signals transmitted through the hippocampus, inducing learning and memory impairments.

The presence of 37 nm GNPs in the hippocampus was also examined by TEM (Fig. 7). Several dark spots were noted in the cytoplasm of neuronal cells (Fig. 7A, 7B, and 7C). Further examination with EDS revealed that these spots were composed of

uranium, possibly due to the heterogeneity of the staining solution (Fig. 7F). Many 37 nm GNPs were detected inside the dendritic structure of brain cells; however, no endocytic structures were associated with 37 nm GNPs in the dendrites. Apparently, the 37 nm GNPs that entered dendrites through free diffusion were excluded from the cell bodies.

There is overwhelming evidence showing that GNPs have negligible toxicity in cultured cells. The in vivo biodistribution has been determined in mice and rats. However, no further evidence regarding the physiological impact to animals has yet been provided. In zebrafish, GNPs exhibited minimal in vivo toxicity with an embryo mortality less than 3%, while silver nanoparticles showed almost 100% mortality. The



difference in toxicity between gold and silver is clearly due more to the unique chemistry of silver and less to the simple reduction in size. If the gold nanoparticles show any size-dependent toxicity, this toxicity will be mild and the particles will be better tolerated than silver nanoparticles. For this reason, the delicate brain function provides an opportunity to prove this hypothesis. These results show that the injection of seemingly nontoxic GNPs can impair the learning and memory of mice at a sufficient dose. The reduction of cognitive ability was associated with the endocytosis of 17 nm GNPs into the neuronal cells in the CA region of the hippocampus. The observation that 37 nm GNPs were found in the extracellular region of the hippocampus was consistent with its inability to impair cognition in mice.

The differential effect of 17 nm and 37 nm GNPs on the cognition of mice indicated that physical diffusion could play a pivotal role. The day 1 and day 14 dose-brain accumulation curves indicated that 17 nm GNPs crossed the blood-brain barrier faster than 37 nm GNPs (Fig. 1). The macroscopic biodistribution of the two GNPs within the brain are indistinguishable from one another. However, the monoamine and acetylcholine profiles were comparable. The microscopic evidence implied that 17 nm GNPs entered into brain tissue and diffused faster within it than 37 nm GNPs. In CARS, the Raman signal of 17 nm GNPs was localized to the CA regions of the hippocampus inside the cluster of neuronal cells, while 37 nm GNPs



were scattered through the peripheral region. The distribution of 17 nm and 37 nm GNPs in the hippocampus implied 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, while 37 nm GNPs were unable to enter neuronal cells, and therefore only caused minimal deficits in learning and memory. The difference in the

effects on cognition of the two GNPs was apparently caused by their difference in cell entry. Although both GNPs caused a global fluctuation in neurotransmitter levels in the brain, differences in their invasive ability into the hippocampus determined the fate of the mice.

The brain is the most delicate and complex organ in animals. Both GNPs studied here affected monoamine profiles in the brain, indicating that brain functions other than learning and memory might be affected by the injection of GNPs. The invading GNPs could also cause abnormal transmission of the electric signals through neurons. It is also possible that engulfment of GNPs may induce an abnormal cellular response, such as apoptosis or an imbalance of intracellular electrolytes. Further experiments will be required to explore the extent and mechanism of the damaging effects of GNPs.

22

## Conclusions

This study showed that the invasion of seemingly nontoxic GNPs can impair the learning and memory of mice. The reduction in cognitive ability was associated with the endocytosis of 17 nm GNPs into neurons of the CA regions of the hippocampus. While GNPs have been widely used for targeting and imaging in drug delivery, this study provides additional insight into the design of drug carriers that deliver molecules to specific areas of the brain.

## Acknowledgements

This study was supported in parts by the National Science Council in Taiwan (grant NSC94-2320-B-009-003) and by Aim for the Top University Plan of the National Chiao Tung University and Ministry of Education, Taiwan..

23

## References

[1] Yih, T. C.; Al-Fandi, M. 2006 J Cell Biochem 97 1184-90

[2] Olivier, J.-C. 2005 NeuroRx 2 108-119

[3] Ferrari, M. 2005 Nat Rev Cancer 5 161-71

[4] Hauck, T. S.; Ghazani, A. A.; Chan, W. C. 2008 Small 4 153-9

[5] Ghosh, P.; Han, G.; De, M.; Kim, C. K.; Rotello, V. M. 2008 Advanced Drug Delivery Reviews 60 1307-1315

[6] Rosi, N. L.; Giljohann, D. A.; Thaxton, C. S.; Lytton-Jean, A. K.; Han, M. S.; Mirkin, C. A. 2006 Science 312 1027-30

[7] Paciotti, G. F.; Myer, L.; Weinreich, D.; Goia, D.; Pavel, N.; McLaughlin, R. E.; Tamarkin, L. 2004 Drug Deliv 11 169-83

[8] Shukla, R.; Bansal, V.; Chaudhary, M.; Basu, A.; Bhonde, R. R.; Sastry, M. 2005 Langmuir 21 10644-54
[9] Connor, E. E.; Mwamuka, J.; Gole, A.; Murphy, C. J.; Wyatt, M. D. 2005 Small 1 325-7
[10] Murphy, C. J.; Gole, A. M.; Stone, J. W.; Sisco, P. N.; Alkilany, A. M.; Goldsmith, E. C.; Baxter, S. C. 2008 Acc Chem Res 41 1721-30
[11] Male, K. B.; Lachance, B.; Hrapovic, S.; Sunahara, G.; Luong, J. H. T. 2008 Analytical Chemistry 80 5487-5493
[12] Patra, H. K.; Banerjee, S.; Chaudhuri, U.; Lahiri, P.; Dasgupta, A. K. 2007 Nanomedicine 3 111-9
[13] Khan, J. A.; Pillai, B.; Das, T. K.; Singh, Y.; Maiti, S. 2007 Chembiochem 8 1237-40
[14] Takahashi, H.; Niidome, Y.; Niidome, T.; Kaneko, K.; Kawasaki, H.; Yamada, S. 2006 Langmuir 22 2-5
[15] Goodman, C. M.; McCusker, C. D.; Yilmaz, T.; Rotello, V. M. 2004 Bioconjug Chem 15 897-900
[16] Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. 2006 Nano Lett 6 662-8
[17] Pan, Y.; Neuss, S.; Leifert, A.; Fischler, M.; Wen, F.; Simon, U.; Schmid, G.; Brandau, W.; Jahnen-Dechent, W. 2007 Small 3 1941-9
[18] Pernodet, N.; Fang, X.; Sun, Y.; Bakhtina, A.; Ramakrishnan, A.; Sokolov, J.; Ulman, A.; Rafailovich, M. 2006 Small 2 766-73
[19] De Jong, W. H.; Hagens, W. I.; Krystek, P.; Burger, M. C.; Sips, A. J. A. M.; Geertsma, R. E. 2008 Biomaterials 29 1912-1919
[20] Sonavane, G.; Tomoda, K.; Makino, K. 2008 Colloids and Surfaces B: Biointerfaces 66 274-280



[21] Bar-Ilan, O.; Albrecht, R. M.; Fako, V. E.; Furgeson, D. Y. 2009 Small 5 1897-910
[22] Kim, J. H.; Kim, K. W.; Kim, M. H.; Yu, Y. S. 2009 Nanotechnology 20 505101
[23] Brown, K. R.; Walter, D. G.; Natan, M. J. 2000 Chem. Mater. 12 306-313
[24] Liu, F.-K.; Ker, C.-J.; Chang, Y.-C.; Ko, F.-H.; Chu, T.-C.; Dai, B.-T. 2003 Jpn. J. Appl. Phys. 42 4152-4158
[25] Hsieh, M. T.; Wu, C. R.; Hsieh, C. C. 1998 Pharmacol Biochem Behav 60 337-43
[26] Christopher J. Addison; Stanislav O. Konorov; Alexandre G. Brolo; Michael W. Blades; Robin F.B. Turner 2009 J. Phys. Chem. C, 113 3586–92
[27] Evans, C. L.; Potma, E. O.; Puoris'haag, M.; Cote, D.; Lin, C. P.; Xie, X. S. 2005

Proc Natl Acad Sci U S A 102 16807-12
[28] Norihiko Hayazawa; Taro Ichimura; Mamoru Hashimoto; Yasushi Inouye; Kawata, S. 2004 Journal of applied physics 95 2676-82
[29] Qian, X.; Peng, X.-H.; Ansari, D. O.; Yin-Goen, Q.; Chen, G. Z.; Shin, D. M.; Yang, L.; Young, A. N.; Wang, M. D.; Nie, S. 2008 Nat Biotech 26 83-90
[30] Chen, Y. S.; Hung, Y. C.; Liau, I.; Huang, G. S. 2009 Nanoscale Research Letters 4 858-864
[31] Myhrer, T. 2003 Brain Res Brain Res Rev 41 268-87
[32] Thomas, S. A.; Palmiter, R. D. 1997 Behav Neurosci 111 579-89
[33] Denenberg, V. H.; Kim, D. S.; Palmiter, R. D. 2004 Behav Brain Res 148 73-8
[34] Spanagel, R.; Weiss, F. 1999 Trends Neurosci 22 521-7
[35] Ogren, S. O.; Eriksson, T. M.; Elvander-Tottie, E.; D'Addario, C.; Ekstrom, J. C.; Svenningsson, P.; Meister, B.; Kehr, J.; Stiedl, O. 2008 Behav Brain Res 195 54-77

25

Table 1 Size distribution and zeta potentials of 17-nm (Sample A) and 37-nm (Sample B) GNPs

The size distribution and zeta potential of the gold nanoparticles was determined by Delsa Nano C (NCTU Instruments Ltd., Hsinchu, Taiwan). GNPs were resuspended in phosphate buffer (pH 7.4, 0.1M) and zeta potential was measured at 25 ┐C.

26

Figure legends

Figure 1. Accumulation of (A) 17 nm GNPs and (B) 37 nm GNPs in the brain. GNPs were injected peritoneally into mice at the indicated doses. Brain tissues were removed 1 day, 14 days, and 21 days after the administration of GNPs. ICP-MS was performed to obtain the concentration of GNPs in brain tissue. Each value represents the average of six independent experiments, and error bars indicate standard deviation.

Figure 2. Learning impairment of passive-avoidance performance induced by scopolamine, 17 nm, and 37 nm GNPs in mice. (A) Mice were randomly assigned to four groups, each containing 8–10 mice: a control group that did not receive any treatment, a positive control group that received scopolamine (1 mg/kg i.p.), the 17 nm GNP-treated group (3.7 mg/kg), and the 37 nm GNP-treated group (3.7 mg/kg). A passive-avoidance test was performed, and the averaged latency time is shown (** $p<0.01$). (B) Dosage response of mice injected with 17-nm GNP in the passive avoidance test. Passive-avoidance test was performed to mice injected with 17-nm

GNPs at doses of 0, 0.4, 0.8, 1.9, 3.7, 7.3, and 14.6 mg/kg. The latency of control group is 180s. The lowest concentration of GNPs with the significantly reduced latency time (138s) comparing to control group is 1.9 mg/kg (* $p<0.05$). The



concentrations below 1.9 mg/kg have no significant effects. Latency time at higher doses is dose-dependent and plateaus rapidly at 7.3 mg/kg.

Figure 3. Fluctuation of monoamine and acetylcholine levels induced by scopolamine, 17 nm GNPs, and 37 nm GNPs in the mouse brain. Immediately after the passive-avoidance test, brain tissues were removed and levels of monoamines and acetylcholine were analyzed. The levels of neurotransmitters and their metabolites are shown in the plots. (A) Norepinephrine (NE) and 4-hydroxy-3-methoxyphenylglycol (MHPG). (B) Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC). (C) 5-Hydroxytyramine (5-HT, serotonin) and 5-hydroxyindoleacetic acid (5-HIAA). (D) Acetylcholine. Each group of columns contains, in sequence, averaged values from the control group, the scopolamine-treated group, the 17 nm GNP-treated group, and the 37 nm GNP-treated group. * indicates $p < 0.05$ and ** represents $p < 0.001$ from the student's t-test.

Figure 4. Global distribution of 17 nm GNPs in mouse brain. (A) Schematic representation of the six areas dissected from the mouse brain and the corresponding 17 nm GNP concentrations based on the results from ICP-MS detection. These values



represent the average of six independent experiments. (B) ICP-MS readings for typical samples obtained from each part of the brain.

Figure 5. CARS microscopy of hippocampi isolated from 17 nm GNP-treated and 37 nm GNP-treated mice. The wavelengths of the pump and the Stokes lasers (Pump = 870 nm and Stokes = 1,064 nm) were tuned to match a Raman shift (~ 2,100 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 shown as red in the final images. The green fluorescence is the auto-fluorescence emitted from the cells of the CA region in the hippocampus. (A), (B): Hippocampi obtained from 17 nm GNP-treated mice. (C), (D): Hippocampi obtained from 37 nm GNP-treated mice. Scale bar=200 μm

Figure 6. TEM images of neuronal cells from the hippocampus of a 17 nm GNP-treated mouse. (A) Entire view. (B), (C), Enlarged areas from (A) showing the invasion of 17 nm GNPs and the surrounding coated pit-like structures in the cytoplasm. (D) Enlarged area from (A) showing the association of GNPs with the

dendrites. (E) is similar to (D) but contains an inset HR-TEM image showing the metallic nature of the black spots. (F) EDS of the selected GNPs in (B).

29
Figure 7. TEM images of neuronal cells from the hippocampus of a 37 nm GNP-treated mouse. (A) Entire view. (B), (C), Enlarged areas from (A) showing the dark spots. (D), (E) Enlarged areas from (A) showing the association of GNPs with dendrites. (F) EDS of the dark spots in (B) identifies these spots to be uranium.

30
Fig. 1

31
Fig. 2

32
Fig. 3

33
Fig. 4

34
Fig. 5

35
Fig. 6

36
Fig. 7

1
Size-dependent impairment of cognition in mice
caused by the injection of gold nanoparticles
Yu-Shiun Chena, Yao-Ching Hungb, Li-Wei Linb, Ian Liauc, Meng-Yeng Honga, and G. Steve Huanga, *
a Institute of Nanotechnology, Department of Materials Science and Engineering, National Chiao Tung University, 1001 University Road, Hsinchu 300, Taiwan (R.O.C.)

bSection of Gynecologic Oncology, Department of Obstetrics and Gynecology, China Medical University and Hospital, 91 Hsueh Shih Rd., Taichung 404, Taiwan, (R.O.C.)
cDepartment of Applied Chemistry, National Chiao Tung University, 1001 University Road, Hsinchu 300, Taiwan (R.O.C.)
*Corresponding author: Institute of Nanotechnology, Department of Material Science and Engineering, National Chiao Tung University, Hsinchu, Taiwan, R.O.C. E-mail address: gstevehuang@mail.nctu.edu.tw, Fax: +886 3 5729912. Tel: +886-3-5131451



Abstract
We explored the size-dependent impairment of cognition in mice caused by the injection of gold nanoparticles (GNPs). GNPs 17 nm and 37 nm in diameter were injected intraperitoneally into BALB/c mice at doses ranging from 0.5 to 14.6 mg/kg. ICP-MS was performed on brain tissue collected 1, 14, and 21 days after the injection. A passive-avoidance test was performed on day 21. Monoamine levels were determined on day 21. The microscopic distribution of GNPs in the hippocampus was examined using coherent anti-Stoke Raman scattering (CARS) microscopy and transmission electron microscopy (TEM).
The results indicated that 17 nm GNPs passed through the blood-brain barrier more rapidly than 37 nm GNPs. Treatment with 17 nm GNPs decreased the latency time, which was comparable to the effect of scopolamine treatment, while 37 nm GNPs showed no significant effect. Dopamine levels and Serotonin levels in the brain were significantly altered by the injection of 17 nm and 37 nm GNPs. GNPs affected dopaminergic and serotonergic neurons. CARS microscopy indicated that 17 nm GNPs entered the Cornu Ammonis (CA) region of the hippocampus, while 37 nm GNPs were excluded from the CA region. TEM verified the presence of 17 nm GNPs in the cytoplasm of pyramidal cells.
In this study, we showed that the ability of GNPs to damage cognition in mice



was size-dependent and associated with the ability of the particles to invade the hippocampus. The dosage and duration of the treatment should be taken into account if GNPs are used in the future as vehicles to carry therapeutic agents into the brain.
Keywords: gold nanoparticles, nanotoxicity, hippocampus, mice, learning impairment, monoamines, transmission electron microscopy.



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 for drug and gene delivery because they possess several unique chemical and physical properties for the transportation and delivery of pharmaceuticals [4-7]. One principal advantage of these carriers is that the gold core is essentially inert and non-toxic. However, the toxicity of these particles due to their small size has yet to be carefully examined.

The cytotoxicity of GNPs has previously been examined [8, 9], and GNPs may or may not be toxic to cell lines depending on the GNP size, surface modifications or the cell type used [10-15]. The cellular uptake of GNPs probably occurs through endocytosis and the largest amount of uptake occurs for 50 nm particles [16]. The size-dependent cellular uptake of GNPs was confirmed in a prostate cancer cell model [17]. Once particles enter cells, certain types of GNPs under 2 nm in diameter are toxic to many cell lines, while larger GNPs exhibit no toxic effects [18]. Although no apparent cytotoxicity has been found, GNP uptake has been associated with damage to the cytoskeleton and cell adhesion [19]. Alternatively, cytotoxicity of GNPs may be modified by coated materials [20]. For example, polyethylene glycol (PEG) coating



greatly improves the biocompatibility and dispersion stability in an in vitro cell model [21].

The tissue distribution of GNPs in rats and mice has been examined in vivo by inductively coupled plasma mass spectrometry (ICP-MS) [22, 23]. GNPs of 10 nm in diameter were present in the liver, spleen, kidney, testis, thymus, heart, lung, and brain; while GNPs larger than 50 nm were largely detected in the blood, liver, and spleen. In particular, GNPs ranging from 10 to 50 nm were found in the brain. In another study, PEG-modified 13 nm GNPs accumulated in the liver and spleen for up to 7 days after injection and induced acute inflammation and apoptosis in the liver [24]. The PEG-coated 20 nm GNPs showed significantly higher tumor uptake in a pharmacokinetics and biodistribution study of nude mice [25]. PEG-modified gold nanorods were found in the blood stream and in the liver [26]. Another group observed that long-term retention of gold nanorods in the liver and spleen does not change the oxidation states of gold [27].

GNPs can pass through the blood-retinal barrier, but retinal toxicity was not observed [28]. It is important to investigate the physiological impact of naked GNPs once these particles enter the brain. This study will provide additional insight into the use of GNPs as carriers for drug delivery to the brain.

## Materials and methods

### Materials

HAuCl4, sodium citrate, NaBH4, HCl, HNO3, H2SO4, H2O2, and other analytical grade chemicals were purchased from Sigma-Aldrich and Fisher in the United States. H2O was obtained at >18 M from a Milli-Q water purification system.

### Preparation of gold nanoparticles

Gold nanoparticles (GNPs) with diameters of 17 and 37 nm were synthesized as previously reported [29, 30]. 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 changing the volume of seed colloid. The solution was stirred for an additional 5–10 min at 0–4 °C. The 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 (Supporting information, Fig. 1S). The potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed GNPs was characterized by zeta potentials (Table 1). Zeta potentials of both



GNPs fell between ±40 and 60 mV, indicating good stability of the colloidal gold in the solution. GNPs were dialyzed against phosphate-buffered saline (pH 7.4) before injection to avoid toxicity from the buffer, such as endotoxin.

### Animal treatment

Animal treatments were performed following “The Guidelines for the Care and Use of Experimental Animals” of National Chiao Tung University in 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 four groups of 8–10 mice, including a control group consisting of mice that did not receive any treatment, a positive control that received scopolamine (1 mg/kg i.p.), a 17-nm GNP-treated group, and a 37-nm GNP-treated group. GNPs were administered in a single dose intraperitoneally. A passive-avoidance test was performed on day 21 after the GNPs were administered. The animals were sacrificed at the end of the experiment by cervical dislocation, after which the brain was isolated and weighed. Excised tissue samples were washed with normal saline and stored at -70 ºC for further assays.

Dose-dependent accumulation of GNPs was performed. GNPs at doses of 0.5 mg/kg, 0.9 mg/kg, 1.8 mg/kg, 3.7 mg/kg, 7.3 mg/kg, and 14.6 mg/kg were



administered. Animals were sacrificed on day 1, 14, and 21. The brain was isolated and weighed. ICP-MS was performed to obtain concentration of gold accumulated in the brain.

Passive-avoidance test [31]

The apparatus consisted of two compartments with a steel-rod grid floor (Supporting information, Fig. 2S; 36 parallel steel rods, 0.3 cm in diameter, set 1.5 cm apart). One of the compartments (48 x 20 x 30 cm) was equipped with a 20-W lamp located in the center of the apparatus at a height of 30 cm and the other was a dark compartment of the same size. The compartments were connected through a guillotine door (5 x 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. A 1-mA scrambled footshock was delivered through the grid floor for 2 s. 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 similar to the training trial, the guillotine door was opened and the step-through latency was recorded. If the mouse did not step through the door after 300 s, the experiment was ended.

Analysis of monoamine and acetylcholine concentrations in the mouse brain

Monoamine levels were determined as previously reported [31]. 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 the maximum setting for 20 s in 10 volume equivalent 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 um), the samples were separated using high performance liquid chromatography (HPLC). Monoamines and their metabolites were separated using HPLC at 30 oC on a reverse-phase

analytical column (ODS-80, 4.6 mm i.d. x 15 cm) and detected by an electrochemical detector (Model ECD-100, Eikom Co., Kypto, 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

10

their metabolites were measured: norepinephrine (NE), 4-hydroxy-3-methoxyphenylglycol (MHPG), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytyramine (5-HT, serotonin), and 5-hydroxyindoleacetic acid (5-HIAA).

Acetylcholine (Ach) levels were determined as previously described [31]. The HPLC system (DSA-300, Eicom) consisted of a detector with a platinum electrode. A guard column and an enzyme column were placed before and after the analytical column (4.6 mm × 160 mm, Eicompak AC-GEL; Eicom), respectively. Isopropylhomocholine was added to the sampling tubes as an internal standard, and the mixture was analyzed using HPLC. The mobile phase was 0.1 M phosphate buffer (pH 8.3), and the flow rate was 0.6 mL/min. Ach levels in the sample were quantified by using the internal standard method.

Statistical Analyses

All data are presented as mean ± SD with a minimum of six mice in each group. Concentrations of biogenic amines and Ach in the mouse brains were analyzed using an unpaired student t-test. The criterion for statistical significance was $p<0.05$ for all statistical evaluations.

11

Inductively coupled plasma mass spectrometry (ICP-MS)

For the total element determinations, standard solutions were prepared by diluting a multi-element standard (1,000 mg/L in 1 M HNO3) 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 the metal-binding proteins that were eluted from the HPLC system were detected by ICP-MS (Perkin Elmer, SCIEX ELAN 5000). The

operating conditions for the machine were as follows: RF power 1,900 W, carrier gas flow 0.8 L/min Ar, and makeup gas flow 0.19 L/min Ar. 197Au was used as the internal standard.

Ex vivo coherent anti-Stoke Raman scattering (CARS) microscopy



Freshly removed hippocampi were dissected into thin slices, approximately 2 mm in thickness, and immersed in a microchamber on a glass slide under PBS for examination. CARS microscopy was performed with a time constant of 3 ms, a scanning area of 300 x 300 μm, a step size of 1 μm, 300 x 300 pixels, a scanning velocity of 1 μm/ms, and a sampling rate of 80 kHz. The 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 (~ 2,100 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 "control" did not show appreciable contrast under the non-resonant condition, whereas the CARS signals were dramatically enhanced, i.e., they appeared as scattered bright spots on the images taken from the GNP-treated specimens. The enhancement presumably resulted from strong scattering by the GNPs and the large third-order polarizability of the GNPs [32-35].

Transmission electron microscopy (TEM)

Small pieces of unfixed tissue were fixed in 2.5% glutaraldehyde with 0.05 M sodium cacodylate-buffered saline (pH 7.4) at room temperature for 2 h. The primary fixation was followed by three 20 min washes with 0.05 M sodium



cacodylate-buffered saline (pH 7.4). The samples were then placed into a 1% OsO4 solution in the same buffer at room temperature for 1 h. OsO4 fixation was followed by three 20 min distilled-water washes and dehydration in acetone. The samples were transferred to 33% and 66% Spurr resin/acetone solutions successively, with a 30-min incubation in each solution. Then, the samples were transferred to 100% Spurr resin, first for 5 h and then they were placed in fresh resin overnight. The samples were cut into 100 nm sections using an ultramicrotome. The grids with ultrathin sections were post-stained with uranyl acetate for 30 min followed by lead for 3 min. After the post-staining procedure, a thin layer of carbon was evaporated onto the grid surfaces. Ultrathin-sectioned material was examined with a Jeol 1400 and a 3200 FS TEM.

Results and discussion

Dose-dependent biodistribution of GNPs in mouse brain

GNPs (17 and 37 nm in diameter) were synthesized according to published procedures [29, 30]. The synthesis was monitored by UV absorbance, and the particle size was examined by electron microscopy (17 ± 1.5 nm and 37 ± 2.1 nm). The potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed GNPs was characterized by zeta potentials (Table 1). Zeta potentials of both GNPs fell between ±40 and 60 mV, indicating good stability of the



colloidal gold in the solution. The purified GNPs were injected intraperitoneally into the BALB/c mice at doses of 0.5 to 14.6 mg/kg. ICP-MS was performed on brains sampled at 1, 14, and 21 days after the injection to evaluate residual GNPs in the brain (Figure 1). GNPs were detected in brain samples one day after the injection. GNPs accumulated rapidly in the first two weeks and continued to increase until the end of the third week. For all dosages, the amount of 17 nm GNPs deposited in the brain was approximately 20% higher than the amount deposited for 37 nm GNPs on days 1 and 14, while the levels were similar on day 21. It is likely that the 17 nm GNPs passed through the blood-brain barrier more readily than 37 nm GNPs, resulting in faster initial accumulation. However, the GNP levels were comparable on day 21. When the dosage of either GNP was higher than 7.3 mg/kg, symptoms of toxicity were noted in the mice at day 21. The treated animals showed fatigue, loss of appetite, changes in fur color, and weight loss. Starting from day 21, the mice showed a significantly camel-like back and a crooked spine. These symptoms were consistent with previous results showing that high doses of GNPs induced multiple abnormalities in mice [36].

GNPs impair learning and memory in mice

To explore if the injected GNPs retarded brain function in mice, particularly learning and memory, we examined the passive-avoidance performance of



GNP-treated mice at a GNP dose of 3.7 mg/kg. The dose was chosen as a minimal dose to avoid lethal effect of GNPs. Previously, we demonstrated that the lethal dose of GNPs was 8 mg/kg/week for four consecutive weeks. However, the current study employed lower doses, which only caused minor symptoms in mice. Scopolamine has the potential to induce amnesia and was applied as the positive control (Fig. 2A). Untreated mice exhibited a latency of 180±9 s. Scopolamine induced amnesia, resulting in an avoidance latency of 92±22 s, a 50% reduction compared to the untreated controls ($p < 0.01$). Although both GNPs caused weakness in mice, an insignificant reduction in latency was observed for the 37 nm GNP-treated mice, while the 17 nm GNP-treated mice showed a latency of 81±25 s in the

passive-avoidance performance test ($p < 0.01$). The latency in the 17 nm GNP-treated mice was comparable to the amnesia caused by scopolamine treatment. Apparently, 17 nm GNPs cause amnesia in mice, while 37 nm GNPs have no effect.
A dose response curve for various concentrations of 17 nm GNP was obtained. The passive-avoidance test was performed on mice injected with 17 nm GNPs at doses of 0, 0.4, 0.8, 1.9, 3.7, 7.3, and 14.6 mg/kg. The lowest concentration of GNPs with a significantly reduced latency time (138±10 s) comparing to the control group was 1.9 mg/kg ($p<0.05$). The concentrations below 1.9 mg/kg had no significant effects. The latency time at higher doses was dose-dependent and plateaued rapidly at 7.3 mg/kg.

16
The monoamine and acetylcholine concentration profiles in the mouse brain were significantly affected by GNPs
Formation and consolidation of learning and memory are associated with the activity of acetylcholinergic, norepinephrinergic, dopaminergic, and serotonergic neurons [37]. Most of these neurotransmitter systems can influence learning and memory in mice. GNP-treatment induced learning impairment, which indicated that GNPs might cause an imbalance of neurotransmitters in the mouse brain (Fig. 3). Norepinephrine negatively regulates the learning and memory process [38]. However, administration of scopolamine, 17 nm GNPs, and 37 nm GNPs did not affect the levels of norepinephrine and its metabolite MHPG. Activation of the dopaminergic system also causes learning impairment [39, 40]. GNP treatment elevated levels of dopamine from 114.5 ng/g brain to 143.6 ng/g brain for the 17 nm GNPs ($p < 0.01$), and to 138.2 for the 37 nm GNPs ($p < 0.05$). Serotonin was significantly reduced from 57.2 ng/g brain to 44.3 ng/g brain ($p < 0.05$) upon treatment with 17 nm GNPs [41]. Overall, GNP-induced learning impairment was correlated with an increase of dopamine and a decrease of serotonin in the mouse brain.

17
The macroscopic distribution of 17 nm and 37 nm GNPs in the brain were indistinguishable
The differential effects of 17 nm and 37 nm GNPs on the cognition of mice implied that the distribution of GNPs in the brain might be size-dependent. ICP-MS was used to detect the distribution of GNPs in the mouse brain. After 21 days of GNP injection at a dose of 3.7 mg/kg, mouse brains were dissected into six parts: the left and right frontal lobes, left and right medial temporal lobes, and the left and right occipital lobes. Both 17 nm and 37 nm GNPs were detected in all parts of the brain at concentrations ranging from 496.2 to 559.5 ng/g brain. The lowest concentration was

found in the occipital lobes (Fig. 4). We were unable to differentiate the macroscopic distributions of 17 nm and 37 nm GNPs in the samples. The ICP-MS results indicated that 17 and 37 nm GNPs were capable of passing through the blood-brain barrier and entering the mouse brain.

CARS microscopy differentiated the local distributions of 17 nm and 37 nm GNPs in the hippocampus

The hippocampus is located in the medial temporal lobe of the brain, belongs to the limbic system, and plays major roles in short-term memory as well as spatial navigation. Since GNP injection impaired learning and memory in mice, the GNPs

18

could have been transported through the blood, across the blood-brain barrier into the brain, and into the hippocampus. To verify the presence of GNPs, the freshly dissected hippocampi were observed using ex vivo CARS microscopy (Fig. 5). GNPs are known to enhance the anti-Stoke Raman signal of nearby amino acids. With proper controls, the enhancement made possible by CARS strongly indicated the presence of GNPs. GNPs were also diffused ex vivo into brain tissues to verify the enhancement of the Raman signal. Localized enhancement of anti-Stoke 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 mouse tissues. The Raman signal of 17 nm GNPs was localized to the Cornu Ammonis (CA) region of the hippocampus inside a cluster of neuronal cells, while 37 nm GNPs were scattered throughout the peripheral region. The distribution of 17 nm and 37 nm GNPs in the hippocampus suggested that the invasion of GNPs into the cluster of neuronal cells in the CA might have caused learning impairment in the 17 nm GNP-treated mice, while 37 nm GNPs were incapable of entering neuronal cells and only caused minimal deficits in learning and memory.

19

TEM revealed that 17 nm GNPs were located in the cytoplasm of hippocampal neurons while 37 nm GNPs were not

TEM was performed to verify the cytoplasmic location of the 17 nm and 37 nm GNPs in the hippocampus 21 days after the injection of GNPs (Fig. 6 and Fig. 7). A total of 72 TEM images were examined. We found that the 17 nm GNPs were located in the cytoplasm of pyramidal cells (Fig. 6A, 6B, and 6C). The Au composition was verified using energy dispersive X-ray spectroscopy (EDS; Fig. 6F), and the gold was also detected using HR-TEM (Fig. 6E). The 17 nm GNPs were found to be associated

with dendrites (Fig. 6D). In particular, the 17 nm GNPs were surrounded by coated pit-like structures in the cytoplasm, leading us to suspect that the 17 nm GNPs entered the cells through endocytosis. However, no endocytosis-related structures were found for the 17 nm GNPs at the dendrites. This result implies that these GNPs entered the dendrites through free diffusion. Additionally, these results suggest that alternative mechanisms for the cell uptake of GNPs occur together as previously reported. The invasion of metallic particles, such as GNPs, into neuronal cells and dendrites could seriously interfere with electric signals transmitted through the hippocampus, thereby inducing learning and memory impairments.

The presence of 37 nm GNPs in the hippocampus was also examined by TEM (Fig. 7). Several dark spots were noted in the cytoplasm of neuronal cells (Fig. 7A, 7B, and



7C). Further examination with EDS revealed that these spots were composed of uranium, possibly due to the heterogeneity of the staining solution (Fig. 7F). Many 37 nm GNPs were detected inside the dendritic structure of brain cells. However, no endocytic structures were associated with 37 nm GNPs in the dendrites. Apparently, the 37 nm GNPs that entered dendrites through free diffusion were excluded from the cell bodies.

There evidence overwhelming showed that GNPs have negligible toxicity in cultured cells. The in vivo biodistribution has been determined in mice and rats. However, no further evidence regarding the physiological impact in animals has been provided. In zebrafish, GNPs exhibited minimal in vivo toxicity with an embryo mortality less than 3%, while silver nanoparticles showed an almost 100% mortality. The difference in toxicity between gold and silver was due more to the unique chemistry of silver and less to with a simple reduction in size. If the gold nanoparticles showed any size-dependent toxicity, this toxicity would be mild and the particles would be better tolerated than silver nanoparticles. For this reason, the delicate functions of the brain provided an opportunity to prove this hypothesis. These results showed that the injection of seemingly nontoxic GNPs can impair the learning and memory of mice at a sufficient dose. The reduction of cognitive ability was



associated with the endocytosis of 17 nm GNPs into the neuronal cells in the CA region of the hippocampus. The observation that 37 nm GNPs were found in the extracellular region of the hippocampus was consistent with the inability of the GNPs to impair cognition in mice.

The differential effect of 17 nm and 37 nm GNPs on the cognition of mice

indicated that physical diffusion could be a key process. The day 1 and day 14 dose-brain accumulation curves indicated that 17 nm GNPs crossed the blood-brain barrier faster than 37 nm GNPs (Fig. 1). The macroscopic biodistribution of the two GNPs within the brain were indistinguishable from one another. However, the monoamine and acetylcholine profiles were comparable. The microscopic evidence implied that 17 nm GNPs entered into the brain tissue and diffused faster than 37 nm GNPs. In CARS, the Raman signal of 17 nm GNPs was localized to the CA regions of the hippocampus inside the cluster of neuronal cells, while 37 nm GNPs were scattered through the peripheral region. The distribution of 17 nm and 37 nm GNPs in the hippocampus implied that the invasion of GNPs into a cluster of neuronal cells in the CA might have caused learning impairment in the 17 nm GNP-treated mice. In contrast, 37 nm GNPs were unable to enter neuronal cells, and therefore caused only minimal deficits in learning and memory. The difference in the effects on cognition of the two GNPs was apparently caused by the difference in cell entry. Although both

22

GNPs caused a global fluctuation in neurotransmitter levels in the brain, the differences in their invasive ability into the hippocampus determined the fate of the mice.

Binding of citrate at the gold surface was dynamic. It is possible that surface modifications that could have replaced citrate on the GNPs could have occurred after injection. Proteins such as albumin, immunoglobulins, complement, fibrinogen, and apolipoproteins bind strongly to nanoparticles once the particles are in body fluids [42]. In particular, binding between complement and immunoglobulin (opsonization) promotes receptor mediated phagocytosis [43, 44]. Binding of plasma protein is important for determining the in vivo biodistribution of nanoparticles. This binding might explain how the injected GNPs passed through blood brain barrier and entered into hippocampus.

The brain is the most delicate and complex organ in animals. Both GNPs in this study affected monoamine profiles in the brain, indicating that brain functions other than learning and memory might be affected by the injection of GNPs. The invading GNPs could also have caused the abnormal transmission of electric signals through neurons. It is also possible that the engulfment of GNPs may induce an abnormal cellular response, such as apoptosis or an imbalance of intracellular electrolytes.

23

Further experiments are necessary to explore the extent of the damaging effects of GNPs.

## Conclusions

This study showed that the invasion of seemingly nontoxic GNPs can impair learning and memory in mice. The reduction in cognitive ability was associated with the endocytosis of 17 nm GNPs into neurons of the CA regions of the hippocampus. While GNPs have been widely used for targeting and imaging in drug delivery, this study provided additional insight into the design of drug carriers that deliver molecules to specific areas of the brain.

## Acknowledgements

This study was supported in part by the National Science Council of Taiwan (Grant NSC94-2320-B-009-003) and by the Aim for the Top University Plan of the National Chiao Tung University and the Ministry of Education in Taiwan.

## References

[1] M. Ferrari, Nat Rev Cancer, 5 (2005) 161-71

[2] J.C. Olivier, NeuroRx, 2 (2005) 108-19

[3] T.C. Yih, M. Al-Fandi, J Cell Biochem, 97 (2006) 1184-90

[4] P. Ghosh, G. Han, M. De, C.K. Kim, V.M. Rotello, Adv Drug Deliv Rev, 60 (2008) 1307-15



[5] T.S. Hauck, A.A. Ghazani, W.C. Chan, Small, 4 (2008) 153-9

[6] G.F. Paciotti, L. Myer, D. Weinreich, D. Goia, N. Pavel, R.E. McLaughlin, L. Tamarkin, Drug
Deliv, 11 (2004) 169-83

[7] N.L. Rosi, D.A. Giljohann, C.S. Thaxton, A.K. Lytton-Jean, M.S. Han, C.A. Mirkin, Science,
312 (2006) 1027-30

[8] E.E. Connor, J. Mwamuka, A. Gole, C.J. Murphy, M.D. Wyatt, Small, 1 (2005) 325-7

[9] R. Shukla, V. Bansal, M. Chaudhary, A. Basu, R.R. Bhonde, M. Sastry, Langmuir, 21 (2005)
10644-54

[10] C.M. Goodman, C.D. McCusker, T. Yilmaz, V.M. Rotello, Bioconjug Chem, 15 (2004)
897-900

[11] J.A. Khan, B. Pillai, T.K. Das, Y. Singh, S. Maiti, Chembiochem, 8 (2007) 1237-40

[12] K.B. Male, B. Lachance, S. Hrapovic, G. Sunahara, J.H. Luong, Anal Chem, 80 (2008)

5487-93
[13] C.J. Murphy, A.M. Gole, J.W. Stone, P.N. Sisco, A.M. Alkilany, E.C. Goldsmith, S.C. Baxter,
Acc Chem Res, 41 (2008) 1721-30
[14] H.K. Patra, S. Banerjee, U. Chaudhuri, P. Lahiri, A.K. Dasgupta, Nanomedicine, 3 (2007)
111-9
[15] H. Takahashi, Y. Niidome, T. Niidome, K. Kaneko, H. Kawasaki, S. Yamada, Langmuir, 22
(2006) 2-5
[16] B.D. Chithrani, A.A. Ghazani, W.C. Chan, Nano Lett, 6 (2006) 662-8
[17] A. Malugin, H. Ghandehari, J Appl Toxicol, 30 (2010) 212-7
[18] Y. Pan, S. Neuss, A. Leifert, M. Fischler, F. Wen, U. Simon, G. Schmid, W. Brandau, W.
Jahnen-Dechent, Small, 3 (2007) 1941-9
[19] N. Pernodet, X. Fang, Y. Sun, A. Bakhtina, A. Ramakrishnan, J. Sokolov, A. Ulman, M.
Rafailovich, Small, 2 (2006) 766-73
[20] Y. Qiu, Y. Liu, L. Wang, L. Xu, R. Bai, Y. Ji, X. Wu, Y. Zhao, Y. Li, C. Chen, Biomaterials,
31 (2010) 7606-19
[21] R.G. Rayavarapu, W. Petersen, L. Hartsuiker, P. Chin, H. Janssen, F.W. van Leeuwen, C. Otto,
S. Manohar, T.G. van Leeuwen, Nanotechnology, 21 (2010) 145101
[22] W.H. De Jong, W.I. Hagens, P. Krystek, M.C. Burger, A.J. Sips, R.E. Geertsma, Biomaterials,
29 (2008) 1912-9
[23] G. Sonavane, K. Tomoda, K. Makino, Colloids Surf B Biointerfaces, 66 (2008) 274-80
[24] W.S. Cho, M. Cho, J. Jeong, M. Choi, H.Y. Cho, B.S. Han, S.H. Kim, H.O. Kim, Y.T. Lim,
B.H. Chung, Toxicol Appl Pharmacol, 236 (2009) 16-24
[25] G. Zhang, Z. Yang, W. Lu, R. Zhang, Q. Huang, M. Tian, L. Li, D. Liang, C. Li, Biomaterials,
30 (2009) 1928-36
[26] T. Niidome, M. Yamagata, Y. Okamoto, Y. Akiyama, H. Takahashi, T. Kawano, Y. Katayama, Y. Niidome, J Control Release, 114 (2006) 343-7

[27] L. Wang, Y.F. Li, L. Zhou, Y. Liu, L. Meng, K. Zhang, X. Wu, L. Zhang, B. Li, C. Chen,
Anal Bioanal Chem, 396 (2010) 1105-14
[28] J.H. Kim, K.W. Kim, M.H. Kim, Y.S. Yu, Nanotechnology, 20 (2009) 505101
[29] K.R. Brown, D.G. Walter, M.J. Natan, Chem Mater, 12 (2000) 306-13
[30] F.-K. Liu, C.-J. Ker, Y.-C. Chang, F.-H. Ko, T.-C. Chu, B.-T. Dai, Jpn. J. Appl. Phys., 42
(2003) 4152-8
[31] M.T. Hsieh, C.R. Wu, C.C. Hsieh, Pharmacol Biochem Behav, 60 (1998) 337-43
[32] C.J. Addison, S.O. Konorov, A.G. Brolo, M.W. Blades, R.F.B. Turner, J. Phys. Chem. C, 113
(2009) 3586-92
[33] C.L. Evans, E.O. Potma, M. Puoris'haag, D. Cote, C.P. Lin, X.S. Xie, Proc Natl Acad Sci U S
A, 102 (2005) 16807-12
[34] N. Hayazawa, T. Ichimura, M. Hashimoto, Y. Inouye, S. Kawata, Journal of Applied Physics,
95 (2004) 2676-81
[35] X. Qian, X.-H. Peng, D.O. Ansari, Q. Yin-Goen, G.Z. Chen, D.M. Shin, L. Yang, A.N. Young,
M.D. Wang, S. Nie, Nature Biotechnology, 26 (2008) 83 - 90
[36] Y.-S. Chen, Y.-C. Hung, I. Liau, G.S. Huang, Nanoscale Research Letter, 4 (2009) 858-64
[37] T. Myhrer, Brain Res Brain Res Rev, 41 (2003) 268-87
[38] S.A. Thomas, R.D. Palmiter, Behav Neurosci, 111 (1997) 579-89
[39] V.H. Denenberg, D.S. Kim, R.D. Palmiter, Behav Brain Res, 148 (2004) 73-8
[40] R. Spanagel, F. Weiss, Trends Neurosci, 22 (1999) 521-7
[41] S.O. Ogren, T.M. Eriksson, E. Elvander-Tottie, C. D'Addario, J.C. Ekstrom, P. Svenningsson,
B. Meister, J. Kehr, O. Stiedl, Behav Brain Res, 195 (2008) 54-77
[42] A.E. Nel, L. Madler, D. Velegol, T. Xia, E.M. Hoek, P. Somasundaran, F. Klaessig, V.
Castranova, M. Thompson, Nat Mater, 8 (2009) 543-57
[43] M.A. Dobrovolskaia, S.E. McNeil, Nat Nanotechnol, 2 (2007) 469-78
[44] D.E. Owens, 3rd, N.A. Peppas, Int J Pharm, 307 (2006) 93-102

Table 1 Size distribution and zeta potentials of 17-nm (sample A) and 37-nm (sample B) GNPs. The size distribution and zeta potential of the gold nanoparticles was determined by Delsa Nano C (NCTU Instruments Ltd., Hsinchu, Taiwan). The GNPs were resuspended in phosphate buffer (pH 7.4, 0.1 M) and the zeta potentials were measured at 25 ┐C.

27

Figure legends

Figure 1. Accumulation of (A) 17 nm GNPs and (B) 37 nm GNPs in the brain. GNPs were injected intraperitoneally into mice at the indicated doses. Brain tissues were removed 1 day, 14 days, and 21 days after administering the GNPs. ICP-MS was performed to obtain the concentration of GNPs in brain tissue. Each value represents the average of six independent experiments, and the error bars indicate standard deviation.

Figure 2. Learning impairment of passive-avoidance performance induced by scopolamine, 17 nm, and 37 nm GNPs in mice. (A) Mice were randomly assigned to four groups, each containing 8–10 mice. The groups included a control group that did not receive any treatment, a positive control group that received scopolamine (1 mg/kg i.p.), the 17 nm GNP-treated group (3.7 mg/kg), and the 37 nm GNP-treated group (3.7 mg/kg). A passive-avoidance test was performed, and the averaged latency time is shown (** $p<0.01$). (B) Dosage response of mice injected with 17 nm GNP in the passive avoidance test. The passive-avoidance test was performed on mice injected with 17 nm GNPs at doses of 0, 0.4, 0.8, 1.9, 3.7, 7.3, and 14.6 mg/kg. The latency of the control group was 180 s. The lowest concentration of GNPs with the significantly reduced latency time (138 s) compared to control group was 1.9 mg/kg

28

(* $p<0.05$). The concentrations below 1.9 mg/kg had no significant effects. The latency time at higher doses was dose-dependent and plateaued rapidly at 7.3 mg/kg.

Figure 3. Fluctuation of monoamine and acetylcholine levels induced by scopolamine, 17 nm GNPs, and 37 nm GNPs in the mouse brain. Immediately after the passive-avoidance test, brain tissues were removed and levels of monoamines and acetylcholine were analyzed. The levels of neurotransmitters and their metabolites are shown in the plots. (A) Norepinephrine (NE) and 4-hydroxy-3-methoxyphenylglycol (MHPG). (B) Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC). (C) 5-Hydroxytyramine (5-HT, serotonin) and 5-hydroxyindoleacetic acid (5-HIAA). (D) Acetylcholine. Each group of columns contains, in sequence, averaged values from the control group, the scopolamine-treated group, the 17 nm GNP-treated group, and

the 37 nm GNP-treated group. * indicates $p < 0.05$ and ** represents $p < 0.001$ from the student's t-test.

Figure 4. Global distribution of 17 nm GNPs in the mouse brain. (A) Schematic representation of the six areas dissected from the mouse brain and the corresponding 17 nm GNP concentrations based on the results of ICP-MS detection. These values

29

represent the average of six independent experiments. (B) ICP-MS readings for typical samples were obtained from each part of the brain.

Figure 5. CARS microscopy of hippocampi isolated from 17 nm GNP-treated and 37 nm GNP-treated mice. The wavelengths of the pump and the Stokes lasers (Pump = 870 nm and Stokes = 1,064 nm) were tuned to match a Raman shift (~ 2,100 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 red in the final images. The green fluorescence is the auto-fluorescence emitted from the cells of the CA region in the hippocampus. (A), (B): Hippocampi obtained from 17 nm GNP-treated mice. (C), (D): Hippocampi obtained from 37 nm GNP-treated mice. Scale bar = 200 μm

Figure 6. TEM images of neuronal cells from the hippocampus of a 17 nm GNP-treated mouse. (A) Entire view. (B), (C), Enlarged areas from (A) showing the invasion of 17 nm GNPs and the surrounding coated pit-like structures in the cytoplasm. (D) Enlarged area from (A) showing the association of GNPs with the dendrites. (E) is similar to (D) but contains an inset HR-TEM image showing the metallic nature of the black spots. (F) EDS of the selected GNPs in (B).

30

Figure 7. TEM images of neuronal cells from the hippocampus of a 37 nm GNP-treated mouse. (A) Entire view. (B), (C), Enlarged areas from (A) showing the dark spots. (D), (E) Enlarged areas from (A) showing the association of GNPs with dendrites. (F) EDS of the dark spots in (B) identifies these spots as uranium.

31

Fig. 1

32

Fig. 2

33

Fig. 3

34
Fig. 4

35
Fig. 5

36
Fig. 6

37
Fig. 7

38
Supplementary information:
Figure 1S. Electron microscopic images of the gold nanoparticles (left: 17 nm, right: 37 nm).
Figure 2S. The apparatus for the passive avoidance test. The 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). The bright compartment (right, 48 x 20 x 30 cm) was equipped with a 20 W lamp. Two compartments (bright and dark) were separated by a wall with guillotine door (5 x 5 cm).