

Citotoxicidad de nanopartículas metálicas en cultivos de células cerebrales

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Resumen

Las nanopartículas (NP) se han investigado por su tamaño diminuto y su posibilidad de llegar a sitios específicos. Se estudian materiales para diseñar nanovectores inocuos. Son pocos los estudios de la exposición a diferentes NP que atraviesan la barrera hematoencefálica (BHE) y de los efectos que tienen sobre células en este tejido.

Objetivo: determinar el deterioro celular en presencia de tres diferentes nanopartículas de origen metálico en cultivo de células cerebrales.

Material y métodos: Realizamos un estudio de viabilidad con MTT y morfológico de cultivos cerebrales (glía y neurona) al contacto con concentraciones de: 10, 50 100 y 250 µg/ml de NP de óxido de silicio (NPSiO₂), oro recubierto con silicio (NPAu@Si) y plata (NPAg)

Resultados: las células cerebrales se ven afectadas en su viabilidad y morfología, siendo más aparente la muerte celular con NPAg, con NPSiO₂ se observa un descenso en la viabilidad de las mismas pero no tan severo como con NPAg, y para las NPAu@Si, se nota un poco más de resistencia incluso a la concentración más alta.

Palabras claves: glía, nanopartículas, neuronas, citotoxicidad.

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Cytotoxicity of metal nanoparticles in brain cell cultures

Abstract

Nanoparticles (NP) have been investigated for their miniscule size and their remarkable to reach specific sites; these materials have been studied to design harmless nano-vectors. However, there are insufficient studies about the effects of the exposure of different NP are able to cross the blood-brain barrier (BBB), over the cells in this tissue.

Aim: To determine the cellular toxicity in the presence of three different nanoparticles of metallic origin in brain in vitro.

Materials and methods: MTT assay and morphological analysis were performed on cultured glia and neurons after treatment with Silica (NPSiO₂), gold-coated with Silicon (NPAu@Si) and silver (NPAg) NP at 10, 50 100 and 250 µg/ml.

Results: Cerebral cells were affected in their viability and morphology after treatment with NPAg. NPSiO₂ treated cells showed a decrease in their viability but not as severe as with NPAg. For NPAu@Si, a slightly higher resistance was observed even at the highest concentration.

Key words: *glia, nanoparticles, neurons, cytotoxicity.*

Introduction

In recent years, nanoparticles (NP) have been studied from various scientific approaches. The characteristics derived from their size, give them particular physicochemical properties different from those presented by the crystalline or bulk material. Particles at the molecular level in a nanomaterial have a maximum size of 100 nm in diameter, they are between one hundred and one thousand times smaller than particles than those found in an amorphous material¹⁻³. NP can access sites of action as they were synthesized. In addition, they can

be functionalized so that they can reach sites with complex environments, as it is the case of cancerous cells, where traditional treatments fail to target exclusively the intended tissue⁴⁻⁸. Nano sciences have become of great importance in the field of health sciences; specifically in the cellular and molecular treatment of diseases, where research is being directed to design and synthesize NP that are able to encapsulate drugs and deliver them to a particular site, across physiological barriers without being metabolized⁹⁻¹¹.

The physicochemical properties of certain materials used in different industries, for example metallurgy, metal mechanics or even electronics have served as a basis for devising some mechanisms to target particular diseases. For this purpose, some nanomaterials have been tested in the brain in order to observe their behavior, distribution and specially their toxicity¹²⁻¹⁸.

The blood-brain barrier (BBB) is an interface that separates the brain from the circulatory system and protects the central nervous system (CNS) from potentially dangerous chemicals and pathological agents; while regulating the transport of essential molecules. Few substances manage to cross the BBB; however, it has been reported that some NP can cross that barrier. Nevertheless, some NP tested both in vivo and in vitro have caused more damage than cellular benefit¹⁹⁻²². The aim of this work is to evaluate changes in morphology and cell viability of primary cultures of brain cells after being treated with three types of NP: SiO₂, Au@Si and Ag.

Materials and methods

i. Synthesis of nanomaterials:

a. Silica Nanoparticles: To obtain the NPSiO₂, a monocrystalline type p wafer doped with boron (Silicon Prime wafers: Part 89), with a resistance of 0.001-0.005 Ω·cm, was carefully fragmented and washed with a 2% HF aqueous solution (Fermont: 01291) followed by an ethanol wash (Golden Bell: HY18101000). The fragments were dried with gaseous nitrogen and placed in a teflon cell with a silver electrode and a platinum counter-electrode. The superficial layer was removed with a HF:Ethanol solution (3:1) in a current density of 350 mA/cm², with an electrical potential of 20

V during 5 seconds. Posteriorly, the wafer was cleaned with a 96% ethanol solution. Following this wash, 1 M KOH was added (Golden Bell: PQ36841) during 5 min in order to remove the superficial porous layer. Finally the wafer was cleaned with 96 % ethanol. For the formation of porous layers, an electrochemical anodizing with alternating pulse intensities was used to create a multilayer structure in a HF:ethanol (3:1) solution. The porous layer of low density was made in the following conditions: 350 mA/cm², 20 V, 150 s. The porous layer of high density was made in the following conditions: 700 mA/cm², 20V, 0.5 s. Three cycles of alternated densities were repeated. The wafer was washed as previously mentioned. The prying of the porous layers was done with 3% HF in ethanol at 70mA/cm², 50 V, 120 s. To carry out the anodizing, power sources connected to a computer with the software Labview were used. The layers recovered were sonicated until particles of the desired size were obtained; they were separated by centrifugation at 14,000 rpm and decantation. The nanoparticles obtained were oxidized with deionized water to obtain NPSiO₂.

b. Silicon-coated gold nanoparticles: 200 ml of 0.5 mM AuCl₄⁻ were poured in a 250 ml round-bottomed flask heated under reflux until boiling while maintaining vigorous stirring. 10 ml of a 38.8 mM sodium citrate solution (pH = 10.5) were rapidly added. After the appearance of a deep red color it was left to boil and stirring for about 1 hour. Subsequently, the temperature was gradually lowered until reaching 25-30 °C. 50 ml of this colloid were taken and 3 ml of 1 mM APTMS were added drop by drop and allowed to stir for 30 minutes. After this, the solution was heated to 90-95 °C and 6 mL of 0.54% sodium silicate was added and maintained at

constant temperature and stirring for 4 hours. The sample was centrifuged at 12,000 rpm for 30 minutes and the precipitate was collected.

c. Silver nanoparticles: 18 mg of Silver Nitrate were added to 100 ml of deionized water in a 250 ml round-bottomed flask, the mixture was let to boil for 20 minutes. After boiling, 2 ml of 0.01 M sodium citrate were added, and let to react for one hour.

ii. Particle size determination

To determine the hydrodynamic diameter and the size distribution of the nanoparticles collected, a light scattering apparatus (Malvern Instruments) was used, performing the dispersion of 100 µl of NP in 1 ml of distilled water.

iii. Primary cultures of cerebral cells

Cells were extracted from 5-7 days old mice brains, by mechanical and enzymatic dissociation of the tissue with trypsin (Gibco: 25300-054). The obtained cells were resuspended in DMEM (Sigma: D5796) enriched with KCl 25 mM (Golden Bell: PI304005), 1 % of streptomycin-penicillin (Sigma: P4333), and 10 % fetal bovine serum (FBS) (Gibco: 16000-044). They were seeded in 35 mm culture plates treated with 2 mM poly-L-lysine (Sigma: 4707). The cells were incubated for 24 hours at 37 °C in 5% CO₂.

Subsequently, the obtained cells were cultured for an additional 24 hours in 96-well plates at a cell density of 10,000 cells per well in the presence of the different nanoparticles at the following concentrations: 10, 50 100 and 250 µg/ml.

iv. Morphological characterization and cellular viability

Morphology was assessed using a light microscope under a 40X objective lens, in order to determine morphological changes on treated cells. To determine cellular viability, the culture medium was replaced with PBS (Sigma: D8537) with 2 mg/ml MTT (Sigma: M2128). The cells were incubated at 37°C, 5 % CO₂ during 35 minutes. After incubation the solution was removed, taking care of not damaging the precipitated tetrazolium crystals. The crystals were dissolved in 100 µl of dimethyl sulfoxide (DMSO) (Sigma: D8418) with vigorous mixing for 60 seconds to ensure a homogeneous dissolution. Mitochondrial activity was determined by absorbance in a spectrophotometer. This test is based on the capacity of healthy mitochondria to reduce tetrazolium [3-(4,5-Dimethyl-2-Tiazolyl)-2,5-diphenyl-2H-Tetrazolium Bromide] to formazan, an insoluble form [1-(4,5-Dimethyl-2 Tiazolyl) 3,5, -Diphenyl formazan], giving a purple coloring.

v. Statistical analysis

All experiments were performed with 4 replicates of each concentrations used in each NP tested. Results are shown as mean ± SD. Statistical significance was assessed with the t-Student test; p values <0.05 were considered statistically significant.

vi. Ethical considerations

The primary cultures were obtained from Balb/C mice, following the regulations for the management of laboratory animals, as well as the guidelines of the ethics committee of the University of Guadalajara.

Results

i. Particle size

The average particle sizes measured by DLS were as follows: NPSiO₂ 53 nm, NPAu@Si 51 nm and NPAg 47 nm.

figure 1 shows the average particle size obtained, versus to the intensity % in which they were located. A low polydispersity was observed (30 and 80 nm in diameter for the nanoparticles).

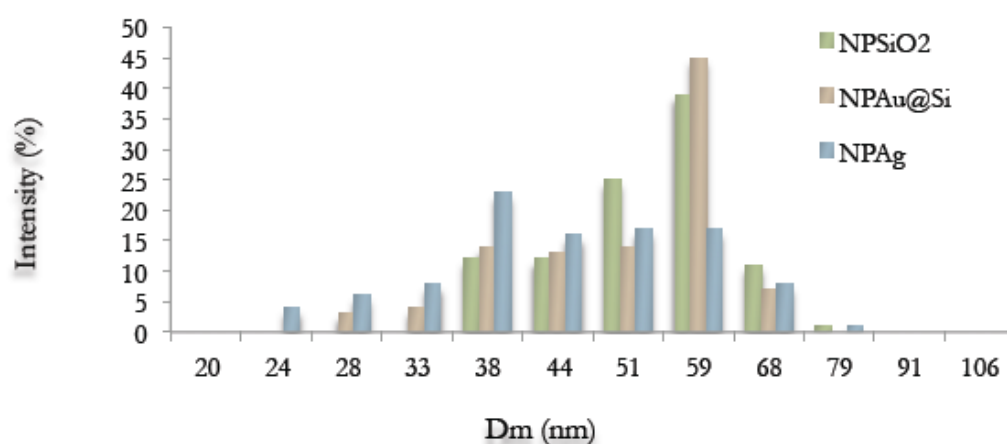


Figure 1. Intensity percentage for each particle size, the average for each nanoparticle is NPSiO₂: 53 nm, NPAu@Si: 51 nm and NPAg: 47 nm

ii. Morphological characterization and cellular viability:

After treating the cell cultures with NP, a microscopic inspection was performed in order to observe any morphological changes of the cells under study.

In figure 2, we show a photographic panel. In the control group cells with well-defined cell

membranes were observed. In the groups treated with NPSiO₂, cells with a well-defined cell membrane were observed at all concentrations; however a possible accumulation of the nanomaterial inside the cell was observed, at concentrations of 100 and 250 µg/ml. Also, cell clumps were observed, being more apparent at 100 µg/ml, this behavior was noticed when

the cells detected a decrease of nutrients in the environment. The presence of detritus was observed at the maximum concentrations used. The groups treated with the NPAu@Si also presented an intact cell membrane. However, it was not possible to determine the presence of accumulated NP inside the cells as observed in the NPSiO₂ treatment.

Cell debris is less apparent, compared to cells that were exposed to NPSiO₂. NPAg treated cells presented structural changes in their cell membrane, and an accumulation of the material was observed. At the concentration of 10 µg/ml, agglomeration of NP was observed, being more apparent in the concentration of 50 µg/ml and 100 µg/ml. However it is not possible to

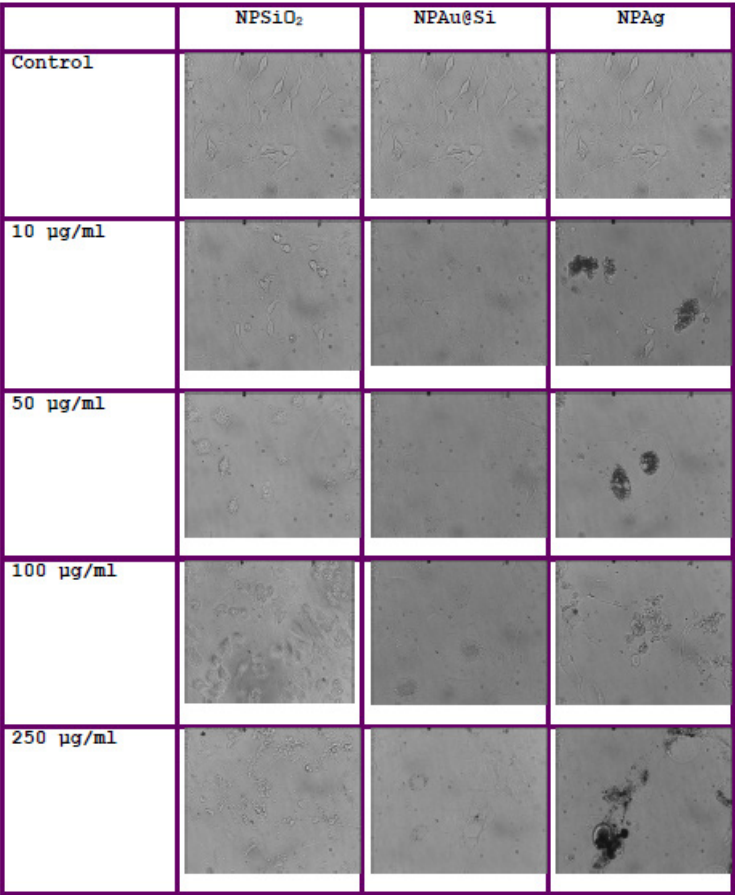


Figure 2. Photographs showing the different morphological changes of brain cells treated with different concentrations of NPSiO₂, NPAu@Si y NPAg.

determine if the NP are located inside or outside the cells. Detritus was observed at 10 $\mu\text{g/ml}$ being more evident at higher concentrations.

After exposure to different concentrations of NP, the percentage of cytotoxicity was determined with the MTT method, in table 1 % viability are presented. A considerably decrease in cell viability was observed in the three types of nanomaterials, even at low concentrations, this effect was dose-dependant. Cells remained slightly more stable on when treated with NPAu@Si; on the other hand, a lower viability was registered in cells treated with NPAg. However, it is noteworthy that treatment with NPSiO₂, cell death was not evident, as there were no observable products of cell death. One possible hypothesis to account for this fact is that the glial cells were still active, collecting cell debris from surrounding dead neurons. Also, it is interesting

Table 1. Viability percentages of primary cultures after treatment with NPSiO₂, NPAu@Si y NPAg.

	NPSiO ₂	NPAu@Si	NPAg
10 $\mu\text{g/ml}$	66	63	55
50 $\mu\text{g/ml}$	58	62	52
100 $\mu\text{g/ml}$	49	56	25
250 $\mu\text{g/ml}$	33	47	22

to note that in the NPAu@Si treatment, lower levels of cell death were noticed, compared to the other two treatment (NPSiO₂ and NPAg).

In figure 3, the graph displays the % of viability for each different concentration of NP. Compared to the control group, a significantly lower cell viability was obtained. Indicating that all three types of NP are toxic to brain cells when exposed *in vitro*.

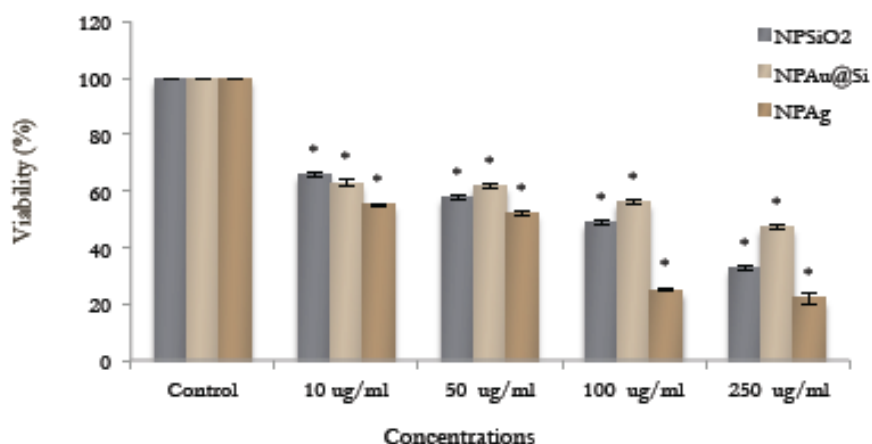


Figure 3. Graph showing the percentages of viability for brain cells in culture at different concentrations of NPSiO₂, NPAu@Si, NPAg. The results represent mean $\pm$ SD of 4 replicas and is expressed en % versus control, *p<0.05.

Discussion and conclusions

The use of metallic nanomaterials in the medical sciences has been increasing due to the characteristics and properties that these materials present at nanometer scale^{18,23}. However, although some are considered inert or biocompatible in certain cell types and tissues, it should be considered whether these materials are capable of crossing the BBB and their toxicity to the brain cells should be determined^{4, 6, 24, 25}.

It is important to note that the three materials used in this work have been previously used as antimicrobial, anti-inflammatory agents or simply as drug vectors in other cell lines without affecting viability^{6, 26-33}.

The MTT assay, is widely utilized as first tool to determine cellular viability, followed by other more specific techniques to determine cellular damage³⁴. Diverse authors have used techniques as: EthD-1²³, LDH³⁵, Tunel Assay³⁶, Annexin V-FITC/PI³⁷ to study cellular viability various cell lines, nevertheless, only a few works regarding brain cell viability are available³⁸.

The results shown in this work, exhibit a high cytotoxicity of the NP tested over brain cells. Further studies are needed to fully determine viability changes in these cells. Previous studies have employed transmission electron microscopy to assess cell morphology, especially in vacuoles³⁸. The particle size of the materials is of vital importance so that they can be considered useful for biomedicine (<100 nm)^{6,15,39}. The NP used in this study ranged between 50-55 nm, however, they could still be considered voluminous, since

there are some reports of NP that have not caused cell damage with diameters smaller than 20 nm⁶. The different synthesis methods employed showed similar particle sizes. Therefore, we cannot conclude whether the toxicity is due to the particle size or the material itself. Due to the fact that the three types of metallic materials caused cytotoxicity to the brain cells after exposure for 24 hs, observing cellular morphological and physiological alterations. Further research aimed at reducing the toxicity is warranted. Strategies such as: reducing particle size, using different types of materials or applying a coating with inert agents that may go unnoticed by the immune system; could improve their viability as drug carriers. As an example, PEG has been used to cover certain nanoparticles, this treatment could be tested in our culture model^{6,40}.

Although the results presented in this work show that the NP at the conditions employed are not innocuous to brain cells, the use of these NP should not be abandoned. Changing variables as already mentioned could minimize the detrimental effects observed. In addition, it would be of interest to know and determine details of the apoptotic pathway triggered in these cells; this data could be extrapolated and applied as a treatment in cell lines responsible for neoplasias such as gliomas.

The search for non-toxic materials that can be used in the area of neurosciences should be continued. SNC-compatible NP have enormous potential applications for the specific transport of drugs, neurotransmitters and hormones, which can not cross the BBB on their own.

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