EFFECT ON NERVE STRUCTURES OF FUNCTIONALIZED GOLD-CHITOSAN NANOPARTICLES OBTAINED BY ONE POT SYNTHESIS

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Abstract: Gold nanoparticles have potential applications in drug delivery, cancer diagnosis and therapy, food industry and environment remediation. However, little is known about their potential toxicity or fate in the environment. In this study we observed significant effects of functionalized gold-chitosan nanoparticles obtained by one pot synthesis on nerve structures of Wistar rats.

INTRODUCTION

The obtaining of metal nanoparticles immobilized in different materials is extremely important in nanoscience and nanotechnology research, since synergistic and bifunctional effects are expected (Mizukoshi *et al*., 2006). There have been many reports regarding the method of metal nanoparticles preparation, such as chemical reduction (Yi *et al*., 1995), photochemical using UV irradiation (Yonezawa *et al*., 1994), sonochemical (Okitsu *et al*., 2007), sonoelectrochemical, etc (Wang *et al*., 2008). Sonochemical method for obtaining reduced gold ions in chitosan solutions involves radical and/or thermal reactions. Chitosan has excellent biocompatible and biodegradable characteristics and is a naturally occurring polysaccharide. Due to the cationic character as polymer and its gel coating properties, the chitosan has been extensively investigated in the pharmaceutical industry for its potential use in the drug delivery development.

Polycationic nature of chitosan results from polycondensation in the presence of anionic molecules. Chitosan has been used (Yao *et al*., 1995) as a protective agent in the preparation of gold nanoparticles and gold salt could be reduced to a zerovalent gold nanoparticles using chitosan, without any additional reducing agent. Thus, gold nanoparticles (Au NPS) with different size distributions have been obtained using chitosan with different molecular weights as an agent of stabilization/reduction. The obtaining of metal nanocomposites with gold nanoparticles has been intensively studied in biology as markers, dyes, catalysts and sensors (Kusumi *et al*., 1993).

The biomedical studies have highlighted a number of effects caused by the combination of chitosan with gold nanoparticles. Sub-acute and acute toxicity studies on male and female rats showed that the chitosan - gold nanoparticles do not produce toxicity in oral administration, showing optimal levels of compatibility (Pokharkar *et al*., 2009). Also, it was shown that Au NPS control the level of glucose, lipids and serum biochemical in mice blood.

The aim of the present study was to investigate the effects of functionalized gold-chitosan nanoparticles, obtained by one pot synthesis, on nerve structures of rats, close related to neurodegenerative conditions.

MATERIALS AND METHODS

Synthesis of gold nanoparticles

The practical grade chitosan (PG), $HAuCl_4 \cdot 3H_2O$ precursor and CH_3-COOH used were obtained from Sigma Aldrich. All solutions were prepared using Milli-Q deionized water (18.2 MΩ resistance).

Numerical molar mass, M_n, gravimetric molar mass, M_w, polydispersity index, PI, and mass distributions were determined by gel permeation chromatography (GPC) using a Varian PL-GPC 120 chromatograph. Thus, for PG, the following values were obtained: M_n = 97,607 g/mol, M_w = 263,836 g/mol, PI = 2.70.

The nanoparticle size optimization was achieved depending on $HAuCl_4 \cdot 3H_2O$ precursor concentration and injected energy into the system by ultrasonic field.

The chitosan stock solution was prepared from 0.1% (0.1 g/L) chitosan in 1% acetic acid (*v/v*). Precursor gold solutions were prepared by mixing 2, 4, 6, 8 and 10 mL of $1 \cdot 10^{-3}$ M $HAuCl_4 \cdot 3H_2O$ stock solution with 38, 36, 34, 32 and 30 mL respectively of 0.1% chitosan stock solution, all these solutions constituting a lot of 5 bottles. The samples were named: 2PG (38 mL PG + 2 mL HAuCl4), 4PG (36 mL PG + 4 mL HAuCl4), 6PG (34 mL PG + 6 mL HAuCl4), 8PG (32 mL PG + 8 mL HAuCl4), 10PG (30 mL PG + 10 mL HAuCl4). Two lots of solutions were stirred and heated by applying an ultrasonic field of 20 kHz with an amplitude of 50% and 80% respectively for 10 minutes, using a Sonoplus Bandelin device.

The micro- and nanophase structure and the average size of lyophilized powder crystallites were investigated by X-ray diffraction with a Shimadzu XRD 6000 diffractometer using CuKα radiation (1.54060 Å). Crystallites size and network constant were calculated using the Topas Academic program.

The micrographic morphology studies by transmission electron microscopy (TEM) were made with a CM100 Philips microscope. For analysis, the nanoparticle solutions were deposited on formvar-coated copper grid.

The nanoparticles average size was determined by visual comparison of TEM micrographies with a standard scale using NIS Elements Basic Research program (NIS-BR). To determine the size distribution it was used the same statistical program.

The nanoparticles size distribution in solution was evaluated with a Malvern Zetasizer Nano ZS, Zen-3500 model, at room temperature. The stability of nanoparticle solutions as a function of average Zeta potential was evaluated by measuring the Zeta potential with the same device, at room temperature.

The characterization of nanoparticles biofunctional properties revealed the biofunctional properties transfer of polymers used as coating agents and stabilizers of nanoparticles.

Animals and treatment

20 male Wistar rats weighing 200-250 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water *ad libitum*. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC). This study was approved by the local Ethic Committee and also, efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were divided into two groups: control group and experimental group. Experimental group was intraperitoneally injected with gold nanoparticles every day for 7 days, respectively.

Coefficients of the brain

After administration of Au NPS (5µg, 8-12 nm) for 7 consecutive days, all animals were weighed, anesthetized (100 mg/kg body weight, ip, Sigma) and then sacrificed. After weighing the body and brains, the coefficients of brain to body weight were calculated as the ratio of tissues: wet weight (mg) to body weight (g) (Ma *et al.*, 2010, Hritcu *et al.*, 2011).

Statistical analysis

Results were expressed as mean ± S.E.M. The results were analyzed statistically by means of the Student's "t" test (T- test: Paired Two Sample for Means). $p<0.05$ was taken as the criterion for significance.

RESULTS AND DISCUSSIONS

Characterization of gold nanoparticles

Depending on the amount of gold precursor solution and energy injected into the system by ultrasonic field, different shades of blue - indigo - violet - brown - yellow can be obtained (Fig. 1). The last sample, 10PG, was stable for a short time, the solution becoming brown to yellow with a brown precipitate.

Fig. 1 - Different colors of gold nanoparticles solution.

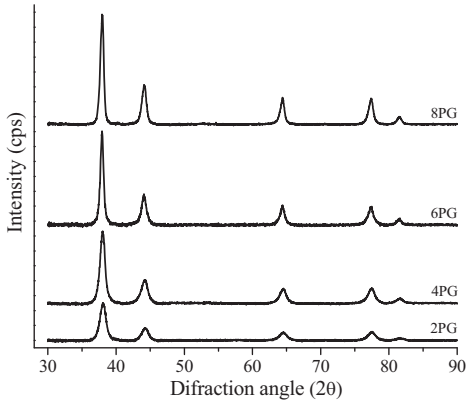

Fig. 2 - Diffractograms for PG with different concentration of $HAuCl_4$.

It is known that the color is strongly dependent on the Au nanoparticles size, the geometrical shape, the mass of polymer covering nanoparticles, etc (Huang and El-Sayed, 2010).

In Fig. 2 are presented the diffractograms for lyophilized powders of 2, 4, 6 and 8 mL 1mM containing gold precursor.

In Table 1 are presented the calculated values for the crystallites size in the direction of Miller planes (hkl), their average size and the network constant (a). Comparing the experimental peaks in Figure 2 with the data specified in the Crystallographic Database we can affirm that the gold particles obtained crystallize in face-centered cubic system (FCC). The crystallites have an average size of 9 – 14 nm and the constant network a = 4.089 Å. For the studies that follow are suitable structures with dimensions as small, so in further tests will be analyzed only the samples 2PG and 4PG.

Table 1. The calculated values for the size of crystallites in the direction of planes (hkl), their average size, and the network constant for the 2PG, 4PG, 6PG, 8PG solutions.

2PG			**4PG**			**6PG**			**8PG**		
Miller (hkl)	Cryst. average size (nm)	a (Å)	Miller (hkl)	Cryst. average size (nm)	a (Å)	Miller (hkl)	Cryst. average size (nm)	a (Å)	Miller (hkl)	Cryst. average size (nm)	a (Å)
1 1 1			1 1 1			1 1 1			1 1 1		
2 0 0			2 0 0			2 0 0			2 0 0		
2 2 0	**9**	**4.084**	2 2 0	**9**	**4.089**	2 2 0	**14**	**4.092**	2 2 0	**13**	**4.091**
3 1 1			3 1 1			3 1 1			3 1 1		
2 2 2			2 2 2			2 2 2			2 2 2		

TEM analysis shows that indeed the obtained nanoparticles have different types of tetrahedral, decahedral, hexagonal, icosahedral, multitwinned, and irregular (Fig. 3).

Size distribution analysis performed with the NIS-BR program (Figure 4) shows that the average sizes of Au nanoparticles is 11.68 nm.

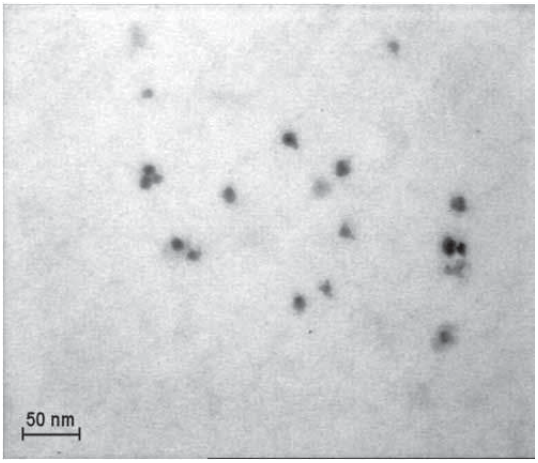

Fig. 3 - TEM micrographs of Au NPS in 2PG sample.

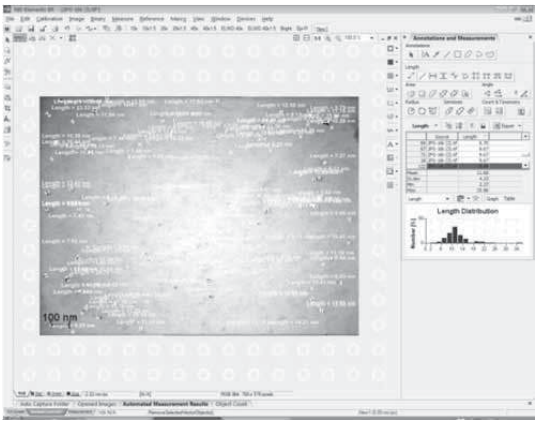

Fig. 4 - Size distributions analyses performed with the NIS-BR program for 2PG sample.

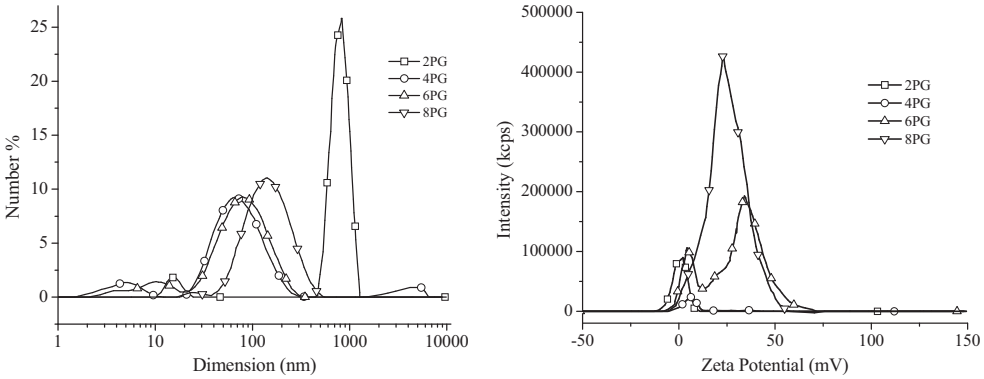

Fig. 5 - The dimension (a) and Zeta potential (b) distributions of Au NPS solutions.

In Fig. 5 are presented the dimentions and Zeta potential distributions for Au NPS coated with chitosan. The Au NPS coated with chitosan average values obtained by LDS measurements are presented in table 2. The values for the size of Au NPS coated with chitosan in aqueous solutions show the same order of magnitude. The average value of Zeta potential is a stability criterion of colloidal solutions (Riddick, 1968). If all the particles in suspension have a large positive or negative potential, they tend to reject them and thus cannot form aggregates. However, if they have small values of Zeta potential, then no force will prevent particle aggregation.

Table 2. The average size and Zeta potential of Au NPS in aqueous solutions.

Sample	Average size (nm)	Average Zeta potential (mV)
2PG	29.9	27.2
4PG	51.5	18.7
6PG	49	29.2
8PG	126	24.9

The results from table 2 show that the stability of obtained Au NPS - chitosan solutions are situated in the category of those who are at the threshold of light dispersion to moderate stability. Experience has shown that these solutions are stable for more than six months. The error in the device decision is due to the fact that there are two distributions: one of the Au NPS nanoparticles coated with chitosan and one of the chitosan micelles (see Fig. 5a).

Coefficient of brain to body weight

Throughout the treatment, animals maintained their weight to normal levels. Daily behavior of animals treated with Au NPS, and food intake, fluid intake was the same as the control animals. Coefficients of brains are shown in table 3 expressed as milligrams (wet weight of tissues)/grams (body weight). No significant differences were found in the body weight of all groups. In the groups treated with Au NPS coefficients of brain significantly decreased compared to control group, suggesting that Au NPS induce degradation of nervous areas involved in memory processes.

Table 3. **Body weight (BW) and coefficients of brain after Au NPS treatment.**

Indexes	Control	Au NPS (5µg)
BW (g)	230 ± 3.16	245 ± 7
Brain/BW (mg/g)	10.5 ± 0,2	8.43 ± 0.5*

Values are means ± SEM, n=10 animals/group, *p <0.05 vs. Control.

CONCLUSIONS

The Au NPS coated with chitosan were obtained by their reduction and nucleation in the chitosan matrix. The nanoparticles were produced in chitosan aqueous solutions by heating under an ultrasonic field, at room temperature. X-ray diffractograms emphasize a structure predominantly FCC with very small grains (crystals): 9 - 10 nm. TEM micrographs show the presence of the NPS in systems, the size and, also, the fact that they have different geometries. AFM micrographs and light scattering analysis on nanoparticles in solution (LDS) show that the

nanoparticles are coated with chitosan. This study suggests that Au NPS are capable of inducing neurotoxicity in rats, close related to neurodegenerative conditions.

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