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ONCOLOGY

GOLD NANOPARTICLES SYNTHESIS AND BIOLOGICAL ACTIVITY ESTIMATION *IN VITRO* AND *IN VIVO*

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The *aim* of the work was the synthesis of gold nanoparticles (GNP) of different sizes and the estimation of their biological activity *in vitro* and *in vivo*. *Materials and Methods:* Water dispersions of gold nanoparticles of different sizes have been synthesized by Davis method and characterized by laser-correlation spectroscopy and transmission electron microscopy methods. The GNP interaction with tumor cells has been visualized by confocal microscopy method. The enzyme activity was determined by standard biochemical methods. GNP distribution and content in organs and tissues have been determined via atomic-absorption spectrometry method; genotoxic influence has been estimated by "Comet-assay" method. *Results*: The GNP size-dependent accumulation in cultured U937 tumor cells and their ability to modulate U937 cell membrane Na$^+$,K$^+$-ATP-ase activity value has been revealed in vitro. Using *in vivo* model of Guerin carcinoma it has been shown that GNP possess high affinity to tumor cells. *Conclusions*: Our results indicate the perspectives of use of the synthesized GNP water dispersions for cancer diagnostics and treatment. It's necessary to take into account a size-dependent biosafety level of nanoparticles .
Key Words: gold nanoparticles, U937 cells, Guerin carcinoma, affinity, biological activity.

Metal nanoparticles are a principally new class of compounds that possess significant biological activity and are potentially perspective for diagnostics and treatment of diseases of different ethiology, especially cancer [1–5].

Gold nanoparticles, in comparison with other metals, are characterized by unique physical, chemical, biological properties and functional activity [6–10]. The nanoparticle size and shape substantially define their properties [11–15]. High affinity to tumor cells, surface modification ability and special optical properties create the basis for effective usage of GNP as vectors for target antitumor drug delivery [16, 17], in cancer phototermal therapy [18–20], as contrasting agents in magnetic resonance and computer tomography [21, 22].

The aim of this work was the synthesis of gold nanoparticles with different sizes and estimation of their biological activity *in vitro* and *in vivo*.

MATERIALS AND METHODS

GNP have been synthesized by Davis' method from the tetrachloroauric (III) acid (HAuCl$_4 \cdot$ 3H$_2$O) ($\geqslant$99.9% trace metals basis, Sigma-Aldrich) [23] and have been characterized by their size using laser-correlation spectroscopy (Zetasizer-3, Malvern Instruments Ltd, UK) and transmission electron microscopy (JEM-1230, JEOL, Japan) methods.

The concentration of obtained GNP was 38.6 µg/ml by metal for each size of preparations.

For *in vitro* experiments U937 (human leukemic monocyte lymphoma) cell line as model of tumor cells has been used. The cell line was obtained from the

Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine. The cell' viability has been estimated by trypan blue exclusion test. The quantity of alive cells was > 90 % in all experiments.

The GNP interaction with tumor cells has been visualized by confocal microscopy method (LSM 510 META, Carl Zeiss, Germany).

U937 cell total membrane fraction isolation has been carried out by the method [24]. Protein content in membrane preparations has been evaluated by the method of Lowry [25].

Na$^+$,K$^+$-ATP-ase activity (E.C. 3.6.1.3) of U937 cell membrane fraction has been measured by method [26] at 37 °C in 1 ml incubation medium (50 мМ Tris-HCl, 5 мМ MgCl$_2$, 100 мМ NaCl, 20 мМ KCl, 3 мМ ATP (pH=7.5)). Membrane aliquot (15–20 µg of protein) was added into incubation medium, incubated for 10 min and stopped by addition of 1 ml 10% trichloracetic acid. The phosphorus content has been measured by Fiske-Subbarow method [27]. For estimation of GNP influence on the enzyme activity the membrane fraction protein (150–200 µg) was mixed with GNP 3 min before the incubation (at concentration range of 0.11–1.1 µg/ml by metal). 20 мМ Tris-HCl buffer has been added in control sample instead of GNP.

For *in vivo* experiments white inbred rats (males and females) with average weight of 180–230 g from vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, have been used.

All experiments with laboratory animals using have been carried out in compliance with "Guide for the Care and Use of Laboratory Animals".

The transplantation of Guerin carcinoma to the laboratory rats has been carried out subcutaneously

on the back using 23% suspension of tumor tissue in physiological solution. The study of GNP influence on the tumor growth *in vivo* has been initiated when tumor size reached ~ 30 mm × 40 mm × 20 mm.

Experimental tumor-bearing animals have been kept at the standard regimen and housed in four groups: control group — 3 animals without treatment; and 3 experimental groups (8 animals per group) treated with single intravenous GNP injection (1 ml of GNP, 38.6 μg/ml by metal) on the basis of 20, 30 and 45 nm preparations.

The euthanasia of animals from control and experimental groups has been performed 1 h after injection (4 animals from each experimental group) and 24 h after administration.

The GNP distribution in tissues (thymus, brain, spleen, liver, kidneys, adrenal glands, lungs, heart and tumor) has been studied using the atomic-absorption spectrometry method [28] on the complex "Graphit -2" (Ukraine). The sensitivity of the method is 6 ng/ml.

The GNP DNA-damaging activity *in vivo* has been estimated by "Comet-assay" method (alkaline gel-electrophoresis of isolated eukaryotic cells) [29]. Cell isolation from liver, kidneys, spleen, bone marrow, intestine and tumor tissue has been performed using standard protocols.

The statistical analysis of the obtained data has been carried out using Student's t-criterion [30]. The differences $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Water dispersions of coagulation-resistant gold nanoparticles have been synthesized for analysis of their activity *in vitro* and *in vivo*. Average sizes of synthesized GNP preparations were 10; 20; 30 and 45 nm according to the data of laser-correlation spectroscopy and transmission electron microscopy. The electron-microscopic image of synthesized 10 nm gold nanoparticles is presented on Fig. 1.
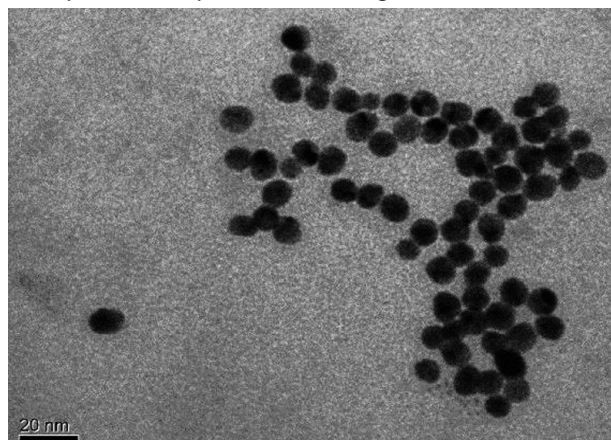

Fig. 1. Electron-microscopic image of synthesized gold nanoparticles with average size of 10 nm (JEM-1230, JEOL, Japan)

The method of synthesis used for the GNP production is based on the conversion of dissolved gold (water solution of tetrachloroauric acid) into insoluble condition with subsequent aggregation and crystallization of insoluble particles which form dispersed phase. The peculiarities of gold nanoparticle structure play an important role in conditions of their contact with different types of biological objects. The different quantity of constituent at-

oms, depending on the size of nanoparticles, bind to the surface: the percentage of surface atoms is higher for smaller particles. The increase of active surface area per mass, changes in interatomic distance and crystal lattice period affect the nanoparticle ability to penetrate into the cell, their biological activity as well as chemical, physical and pharmacological properties [31, 32].

High level of the GNP accumulation in U937 tumor cells has been determined by confocal microscopy by layer-by-layer scanning (Fig. 2).
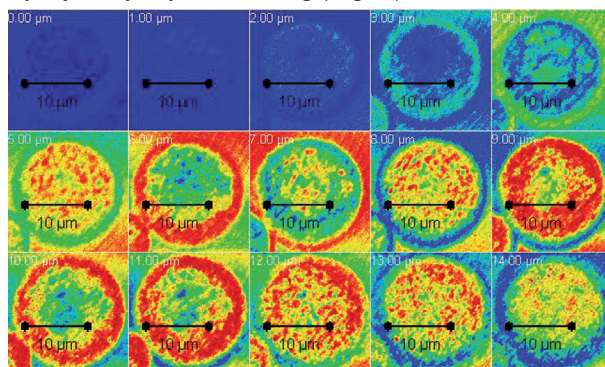
Fig. 2. Confocal-microscopic image of U937 tumor cells (cell concentration is 10^6 cells/ml) after 3 min incubation with 30 nm gold nanoparticles at the concentration of 12.7 μg/ml by metal. Z-scanning with 1 μm step; GNP maximum accumulation is presented as red staining (LSM 510 META «Carl Zeiss», Germany)

The most effective GNP accumulation by U937 tumor cells has been observed for 20 and 30 nm gold nanoparticles.

In vitro biological activity of synthesized gold nanoparticles is estimated through measuring the values of Na^+,K^+-ATP-ase and Mg^{2+}-ATP-ase activities of membrane fraction of U937 cells treated with GNP. The obtained results have demonstrated the dependence of such activity from nanoparticles size (Fig. 3, curves 1–4). GNP with average size of 10 nm in all studied concentrations (0.11–1.1 μg Au/ml) inhibited the enzyme activity by 70% in comparison with control cells (curve 1), GNP with 20 nm diameter — by 20 % (curve 2). At the same time GNP with average size 30 nm and 45 nm in concentration range 0.11–1.10 μg Au/ml stimulated Na^+,K^+-ATP-ase activity by 30–40 % (curve 3), and 20–40% (curve 4), respectively.
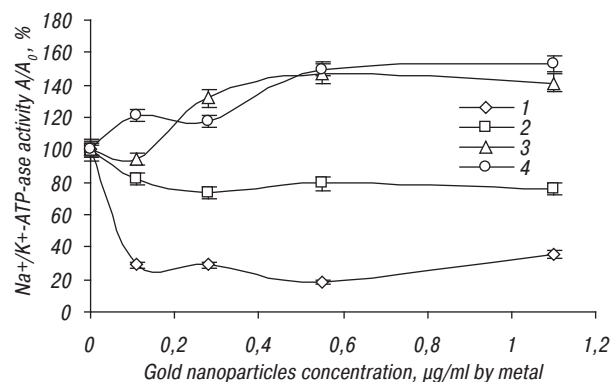
Fig. 3. Changes of U937 tumor cell membrane fraction Na^+,K^+-ATP-ase activity value (A/A_0, %) upon the influence of gold nanoparticles with average sizes: 1–10 nm, 2–20 nm, 3–30 nm, 4–45 nm. (M±m; n=5, $p < 0.05$ compared to control — A_0). Native Na^+,K^+-ATP-ase activity value is considered as 100 % (control)

Thus, in GNP concentration range of 0.11–0.28 µg Au/ml (45nm) we have registered 20% elevation of the enzyme activity, while in concentration range 0.28–0.55 µg Au/ml GNP this value increased from 20% to 40% and was equal to 40% in concentration range of 0.55–1.10 µg Au/ml.

However, treatment of U937 cells with GNP (in all investigated sizes) has no significant influence on Mg^{2+}-ATP-ase activity of cell membrane fraction.

In vivo study of GNP biological activity after their intravenous injection is important for estimation of GNP perspective usage in cancer diagnostics and therapy. That's why we have estimated the patterns of GNP distribution and accumulation in organs and tissues of experimental animals.

Via atomic absorption spectrometry the GNP distribution and accumulation in organs and tissues of experimental normal animals and Guerin carcinoma bearing animals have been studied. For gold content analysis thymus, brain, spleen, liver, kidneys, adrenal glands, lungs, heart and tumor tissue of experimental animals have been examined 1 and 24 h after single GNP i.v. injection. It has been revealed that the gold is not present in studied organs of normal experimental animals either in 1 h nor 24 h after GNP injection. It may be suggested that tumor influences gold nanoparticles distribution in tumor-bearing organism resulting in their accumulation in non tumor tissue in contrast to the non-tumor-bearing host.

However, in studied organs of tumor-bearing animals the picture was different. 20 nm GNP distribution and accumulation in different organs of experimental animals are demonstrated in Table 1. In tumor bearing animals 1 h after GNP injection gold content values were as follows: in brain — 26.09±3.15 ng Au/g tissue, kidneys — 51.40±6.23ngAu/gtissue, spleen — 60.00±5.78ngAu/gtissue, and liver — 37.60±4.07 ng Au/g tissue.

Table 1. Accumulation of GNP with average size of 20 nm in organs and tissues of Guerin carcinoma bearing animals after intravenous injection of nanoparticles (1 ml)

Target organ	Concentration of gold, ngAu/g tissue		
	Control group	1 h after GNP injection	24 h after GNP injection
Tumor	-	78.90±5.27	31.70±2.69
Thymus	-	-	-
Brain	-	26.09±3.15	-
Lungs	-	-	-
Heart	-	-	-
Adrenal glands	-	-	-
Kidneys	-	51.40±6.23	-
Spleen	-	60.00±5.78	-
Liver	-	37.60±4.07	12.70±1.92

Note: "-" – gold content below detection limits (M ± m; n = 4, p < 0.05).

In 24 h after GNP injection the level of gold accumulation in liver, spleen and kidneys has been substantially decreased and amounted to 12.7±1.92 ng Au/tissue for liver and was absent in spleen and kidneys. These data demonstrate the principal role of these organs in detoxication and clearance of GNP from the organism.

The peculiarities of GNP accumulation in brain of tumor-bearing animals have shown their ability

to penetrate through hematoencephalic barrier what can be used in diagnostics and target therapy.

The highest level of the gold accumulation (78.90±5.27 ng Au/g tissue), comparatively with other organs, has been registered in tumors in 1 h after GNP injection, while in 24 h it amounted to 31.7±2.69 ng Au/g tissue, what was twice higher than its residual concentration in liver.

Thus, the GNP high clearance rate in the conditions of their i.v. injection to normal animals has been demonstrated while as GNP predominant accumulation has been determined in tumor tissue at both time points (1 and 24 h after intravenous injection).

GNP usage in diagnostics or therapy should be validated through their biosafety marker tests *in vitro* and *in vivo*. One of most sensitive marker tests of biosafety is genotoxity test, which reveals agent DNA-damaging influence. For this purpose we have used "Comet assay" method.

The GNP genotoxicity, depending on their size, has been analyzed earlier [33, 34] *in vitro* and *in vivo.* The 20 nm GNP genotoxic influence on kidney cells of tumor-bearing rats in 1 h after single GNP intravenous injection has resulted in 24.04% of DNA in comet tail versus 0.24% for negative control (Table 2, Fig. 4). These data evidence on potential risk of 20 nm GNP injection for normal organs.

Table 2. GNP genotoxicity evaluation in organs and tissues of Guerin carcinoma-bearing animals 1 h after intravenous injection of nanoparticles

Target organ	% of DNA in comet tail, negative control	% of DNA in comet tail in GNP-administered animals		
		20 nm	30 nm	45 nm
Liver	0.27	0.44	0.32	0.35
Kidneys	0.24	24.04	0.27	0.33
Spleen	0.23	0.27	0.27	0.27
Tumor	0.92	apoptosis	0.91	0.93
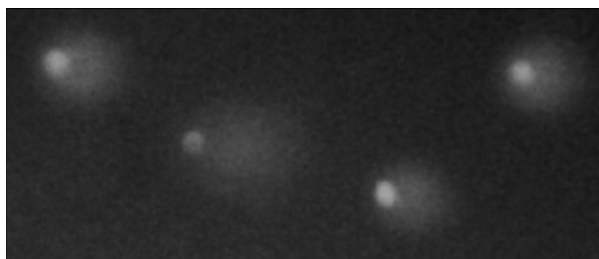
Fig. 4. Electrophoretic image of kidney cell damaged DNA (DNA-comet) 1 h after i.v. injection of 20 nm gold nanoparticles to tumor-bearing animals

1 h after 20 nm GNP injection the apoptosis rate of tumor cells yielded up to 80%. It is the evidence of total tumor cells death.

The 30 and 45 nm GNP injection didn't cause DNA damage in organs and tumor tissue of experimental animals in 1 h after i.v. injection. In other words, 30 and 45 nm nanoparticles revealed biosafety in such test.

In 24 h after i.v. injection of 20, 30 and 45 nm GNP there has been registered no genotoxic influence (Table 3).

Table 3. GNP genotoxicity evaluation in organs and tissues of Guerin carcinoma-bearing animals 24 h after intravenous injection of nanoparticles

Target organ	% of DNA in comet tail, negative control	% of DNA in comet tail in GNP-administered animals		
		20 nm	30 nm	45 nm
Liver	0.31	0.41	0.43	0.42
Kidneys	0.29	0.57	0.52	0.56
Spleen	0.25	0.25	0.27	0.32
Tumor	0.71	0.83	0.77	0.71

So, the high specific size-dependent biological activity *in vitro and in vivo* of synthesized water dispersions of GNP has been revealed. *In vitro* data show that synthesized gold nanoparticles possessed by expressed size-dependent modulation of membrane Na^+,K^+-ATP-ase activity in U937 tumor cells. *In vivo* results indicate that gold nanoparticles possess high affinity to tumor cells after their i.v. injection to experimental animals. These *in vitro* and *in vivo* results point on the perspectiveness of GNP use in cancer diagnostics and treatment, although it is necessary to take into account the size-dependent biosafety level of GNP concerning normal organs.

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