Investigation of Biological Safety of Nanosubstances and Nanomaterials

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Abstract

Results of the toxicological investigation in vivo and in vitro of nanosubstances and nanomaterials obtained at the Institutes of the Siberian Branch of the RAS are presented. It was established that, on the basis of acute toxicity value, the nanopowders of the oxides of aluminium, zinc, titanium, and carbon materials may be related to the class 3 (moderately toxic) or the class 4 (low toxicity), while nanopowders of copper correspond to the class 2 of toxicity. It is demonstrated through the in vitro investigation involving titanium nanopowder as an example that metal nanopowders can be highly toxic for various primary (healthy) cells but cause no death of cancer cells.

Key words: metal nanopowder, toxicity, in vivo, in vitro

INTRODUCTION

Nanotechnology is today recognized as the priority direction of the scientific and technological development on which first of all the national safety of Russia depends. The use of nanosubstances in the production of new nanomaterials, as well as new medical preparations on their basis, requires ascertainment of the action of nanosubstances on biological objects. Evidently, the effects of artificial chemical compounds and nanomaterials can be either momentary or delayed [1]. Thus, the development of nanotoxicology as one of the important subdisciplines of nanotechnology is very urgent. In addition, timely complex investigation of nanoparticles and nanomaterials will allow adequate evaluation of the potential danger of nanoproducts and nanotechnologies, and thus the health of population and safety of environment will be provided.

It is necessary to establish the active feedback between the medical and technological aspects of the application of new nanomaterials at the early stages of their development, which will allow one to reveal their toxic and other hazardous effects in time. Evaluation of the toxicity of new nanomaterials and nanoparticles using laboratory animals is the key stage in the determination of the risk for the application of nanotechnologies [2, 3]. The experiments of this kind, if carried out in full,
are very expensive, labour-intensive and time-consuming, so the methods based on cell test objects are to be introduced as well, for the evaluation of potential danger of a large number of already developed artificial nanoparticles and nanomaterials, as well as those under development. This will allow one to save substantial funds for selecting the most promising and safe directions of the development of nanomaterials and nanotechnologies.

In this connection, we carried out primary toxicological investigations of nickel, iron, copper, titanium and aluminium oxide powders, carbon nanomaterials of different morphologies, both in vitro and in vivo.

**EXPERIMENTAL**

**Physicochemical characteristics of the studied materials**

**Nanopowders.** Nickel nanopowder differing by particle size and the thickness of the protective carbon shell were obtained at the Institute of Solid State Chemistry and Mechanochemistry of SB RAS (Novosibirsk) using the following methods:

- reduction of nickel formate with benzyl alcohol: Ni-0369 lot with the average particle size about 500 nm and lot Ni-0376 with average size of powder particles about 100 nm;
- reduction of the complex compounds of nickel with hydrazine carboxylic acid in ethylene glycol: Ni-0372 lot with average particle size 20–40 nm;
- mechanochemical grinding of nickel in mixture with soot: Ni/C lot; nickel nanoparticles, each particle coated with graphite layer 5–10 nm thick;
- thermal decomposition of nickel formate: Ni-t/d lot, the size of particles of the final product is about 50 nm.

Aluminium oxide, iron, copper, titanium powders were obtained at the Institute of the Problems for Chemical and Energetic Technologies, SB RAS (Biysk) by means of electric explosion of the metal wire (EEW) [4]. Physicochemical characteristics of nanopowders are listed in Table 1.

**Carbon materials**

**Carbon nanomaterials** differing to a high extent by their structure and morphology were obtained at the Boreskov Institute of Catalysis, SB RAS: carbon nanotubes (CNT), carbon nanotubes (CNT), carbon nanofibres (CNF), nitrogen-containing CNF (N-CNF), amorphous microporous carbon (AMCM) and nitrogen-containing AMCM (N-AMCM).

Carbon nanotubes, CNF and N-CNF were synthesized through the catalytic decomposition of hydrocarbon mixtures (methane, ethane, ethylene, and ethylene–ammonia) on metal catalysts. The resulting materials are mesoporous, they have ordered graphite-like structure with specific surface up to 350 m$^2$/g. The diameter of tubes is about 20–30 nm (Fig. 1).

Amorphous microporous carbon and N-AMCM were synthesized through carbonisation of various organic and nitrogen-containing organic precursors. The obtained samples are characterized by high specific surface (up to 3200 m$^2$/g) and micropore volume up to 1.88 cm$^3$/g. Investigation by means of TEM showed that the structure of AMCM and N-AMCM (1–17 %) is represented by bent graphite layers located at a distance of about 1 nm with respect to each other.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Al (Alex$^{TM}$)</td>
</tr>
<tr>
<td>Average particle size, nm</td>
<td>160</td>
</tr>
<tr>
<td>Mass fraction of active metal, % (Al$_2$O$_3$ 7–9)</td>
<td>90–92</td>
</tr>
<tr>
<td>Specific surface, m$^2$/g</td>
<td>15.5</td>
</tr>
</tbody>
</table>
It was established with the help of XPES that nitrogen in N-CNF is present in two major states: pyridine-like \( (E_b = 398.5 \text{ eV}) \) and graphite-like \( (E_b = 400.8 \text{ eV}) \). In turn, in N-AMCM nitrogen is present in several forms: pyridine-like \( (E_b = 398.6 \text{ eV}) \), pyrrole \( (E_b = 400 \text{ eV}) \) and graphite-like \( (E_b = 400.9 \text{ eV}) \) (III) [5–7].

Methods of in vivo studies

Determination of the toxicological parameters of nanosubstances and nanomaterials was carried out with white outbred mice with the body mass of 20–25 g submitted by the Laboratory of Experimental Animals of the Institute of Cytology and Genetics, SB RAS. The animals were kept in accordance with the rules accepted by the European Convention on the Protection of Vertebrate Animals (Strasbourg, 1988). The animals were kept in Velaz standard plastic cages \((4 \times 25 \times 14 \text{ cm})\) on a mat of small wood chips. Temperature of the air in the vivarium was 20–23 °C, humidity not more than 50 %, air exchange volume \((\text{extract/input})\) \(8:10\), light conditions \((\text{day/night})\) \(1:1\). The animals were fed with standard granulated compound feed with mineral and vitamin additives, and grain mush.

Experimental groups were formed of 8–10 individuals with identical mass.

The toxicity of nanopowders and nanomaterials was determined after intragastric introduction. A solution in the form of 3 % starch suspension was introduced through a tube into stomach in the amount of 0.2 mL/10 g of body mass. The duration of observation was 14 days since the moment of introduction. The average lethal doses were calculated using Karber method [8].

Recorded parameters. The state of animals was evaluated from their appearance, behaviour, and also with the help of integral parameters: changes of body mass \((\text{OHAUS balance, USA})\), body temperature \((\text{LabLinc electrophysiological complex, Coulbourn Instruments, USA})\), spontaneous motor activity and investigative reaction \((\text{automatic recording of motions in TruScan chamber, Coulbourn Instruments, USA})\). After the experiment was over, autopsy was carried out, with the macroscopic pathomorphological examination of the internals for the purpose of evaluating the toxic effect of introduced substances.

Methods of in vitro studies

Investigations in vitro were carried out to study the cytotoxic and stimulating action of a number of nanomaterials on the primary and long–cultivated human cells.

Primary endotheliocytes extracted from the umbilical vein of newborn babies, primary fibroblasts from gum tissues, the cells of cervical adenocarcinoma HeLa and breast cancer cells MCF-7 were taken as models. For the quantitative evaluation of the cytotoxic action of nanomaterials, we used the MTT test based on the ability of mitochondrial dehydrogenases to convert water-soluble 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into formazan which crystallizes inside the cell. The transfer of formazan into solution with the help of suitable organic solvents such as dimethyl sulphoxide (DMSO) or...
isopropanol, followed by photometric examination, allows one to perform exact comparison of the change of the optical density of solution, related to the reference, with the change of the number of viable cells; in cytotoxicity studies, specific death of cells induced by a cytotoxic agent can be thus estimated. The minimal number of cells that can be detected in a well of a 96-well plate is 25 thousand. To determine the accuracy of the method, we measured the relative number of cells in 10 repetitions using 5 and 10 thousand cells per one point (a well of the 96-well plate). It was shown that the accuracy of measurement is characterized by the relative error of 5 %. A minimal detectable difference in the number of living cells (or minimal reliably detected number of dead cells) in the case when 5 to 12 thousand cells per a well of the 96-well plate are used in the analysis is 5 % (\( p = 0.05 \)) (calculated as the confidence interval for the average value).

For a quantitative estimation of the stimulating action of nanomaterials, we used the test aimed at measurement of the level of interleukin-6 (IL-6), one of the major anti-inflammatory cytokines.

**RESULTS AND DISCUSSION**

**Determination of the toxicity of nickel nanopowder**

Attention to nickel nanopowders is connected with broad outlooks for their use in various branches of industry, including the production of multiplayer capacitors, diffusion welding of the materials differing in nature, in the production of ferrites, magnets and heat resistant alloys, filtering elements, accumulator and welding electrodes, composite materials etc. Along with silver and copper powders, nickel nanopowders belong to the most essential ones at the market of nanometals. At the same time, judging from the extent of action on a human organism and the ability to cause remote biological consequences, nickel powder belongs to the substances of the first, the highest class of danger. In this connection, investigation of the toxic properties of these substances is essential for the provision of safety for manufacturers and for consumers of these powders [8].

To study the toxicity, nickel nanopowders Ni-0369, Ni-0376, Ni-0372, Ni/C, Ni-t/d were introduced into mice once in the form of 3 % starch suspension in the doses of 500, 1000, 2000, 2500 mg/kg.

Analysis of the data obtained with nickel nanopowders Ni-0369, Ni-0376, Ni-0372, Ni/C showed that single intragastric introduction in the doses 500 to 2500 mg/kg does not lead to the death of animals. Their general action is expressed as insignificant suppression of the central nervous system in the case of the large dose. The average lethal dose for these samples after intragastric introduction (LD50) for mice exceeds 200 mg/kg. Only for the Ni-t/d sample

| TABLE 2 |
| Effect of metal nanopowders on the parameters of motor and emotional behaviour of animals |

<table>
<thead>
<tr>
<th>Nanopowders</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>FP CENTRE DIST</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose: 10 mg/kg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>10.9±0.8</td>
<td>106±1.04</td>
<td>330.5±13.3</td>
<td>2.8±0.1</td>
<td>14±1.0</td>
<td>85.9±11</td>
<td>8.8±1.1</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>11.5±0.8</td>
<td>106.9±1.3</td>
<td>297±11.1</td>
<td>2.5±0.1</td>
<td>12±1.3</td>
<td>113.3±8.1</td>
<td>9.9±0.8</td>
</tr>
<tr>
<td>Copper</td>
<td>11.6±1.1</td>
<td>105.9±1.3</td>
<td>293.8±23.3</td>
<td>2.4±0.2</td>
<td>14.1±1.3</td>
<td>104±13.7*</td>
<td>12.4±1.5</td>
</tr>
<tr>
<td>Iron</td>
<td>10.8±1.03</td>
<td>105.5±1.7</td>
<td>277±18.9*</td>
<td>2.3±0.2</td>
<td>14.5±1.7</td>
<td>126±9.9*</td>
<td>6.5±1.2</td>
</tr>
<tr>
<td>Titanium</td>
<td>11.5±0.6</td>
<td>105.3±1.0</td>
<td>273±12.8*</td>
<td>2.3±0.1</td>
<td>14.8±1.0</td>
<td>98.6±10.5</td>
<td>8.8±1.9</td>
</tr>
<tr>
<td><strong>Dose: 500 mg/kg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>13±0.89</td>
<td>101.2±0.9</td>
<td>288.5±27.3</td>
<td>236</td>
<td>18.8±0.9</td>
<td>–</td>
<td>12.8±2.1</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>15±1.18</td>
<td>97.8±1.9</td>
<td>210±16.7*</td>
<td>1.68*</td>
<td>22.2±1.9</td>
<td>–</td>
<td>10.2±1.0</td>
</tr>
<tr>
<td>Iron</td>
<td>15.5±1.48</td>
<td>94±4.1</td>
<td>226.6±25.2</td>
<td>1.83</td>
<td>26±4.1</td>
<td>–</td>
<td>9.8±1.1</td>
</tr>
<tr>
<td>Titanium</td>
<td>17.5±0.89*</td>
<td>88.5±3.5*</td>
<td>191±19.9*</td>
<td>1.55*</td>
<td>31.5±3.5*</td>
<td>–</td>
<td>6.5±2.1</td>
</tr>
</tbody>
</table>

*Note. A – number of motor acts, B – time of activity, s; C – distance travelled, cm; D – motion velocity, cm/s; G – time spent in stands, s; H – number of studied holes; I – time of investigative reaction, s.

*p < 0.05 for reference group.
obtained by thermal decomposition of nickel formate the average lethal dose was somewhat lower and turned out to be less than 1000 mg/kg: the toxic effect of poisoning was observed after the introduction of nanopowder in the dose of 700 mg/kg.

The behaviour and state of experimental animals corresponded to the background parameters within the whole range of doses. No statistically significant changes of body temperature in comparison with the reference group were observed; an increase in body mass was uniform in all the groups. The introduction of all the samples of nickel powder did not cause any substantial effect on the state of the central nervous system of the experimental animals.

On the basis of the macroscopic pathomorphological analysis of internal organs 14 days after the introduction of nickel nanopowder, no pathological changes of the organs in thoracic and abdominal cavities were revealed.

### Toxicity of iron, copper, titanium and aluminium oxide nanopowders

It is known that metal and metal oxide nanoparticles are interesting as biologically active materials. In particular, it is proposed to use nanocopper in post-infarction state and as an antibacterial agent. Nano-iron is wound healing agent. These metals possess cytostatic activity [9]. However, no integrated studies (in vivo and in vitro) of the toxicity of nanomaterials and nanometals had ever been performed. At the same time, aluminium, iron, nickel, titanium and zinc are widely studied in composite materials to make promising explosive compositions and mix solid propellants; the knowledge of toxicity is also necessary in these applications.

In the next series of experiments, we determined the toxicity of iron, copper, titanium nanopowders and aluminium oxide nanopowder after intragastric introduction into mice in the doses of 200, 500, 1000, 2000, 2500 mg/kg. Examination of the toxicity of these nanopowders showed that their single intragastric introduction in the doses 500 to 2500 mg/kg did not cause death of animals; the general action was expressed as insignificant suppression of the central nervous system in the case of the large dose. Single introduction of copper within the dose range 200 to 400 mg/kg caused death of animals. For nanopowders of aluminium oxide, iron and titanium, the average lethal dose after intragastric introduction (LD₅₀) for mice exceeded 200 mg/kg; for copper nanopowder it was 225 mg/kg.

The behaviour and state of the experimental animals corresponded to the background parameters within the whole dose range. No statistically significant changes of body temperature with respect to the reference group were detected; mass increase was uniform in all the groups.

Investigation of the effect of metal nanopowders on the motor and emotional activity of animals after single intragastric introduction in the doses of 10 and 500 mg/kg showed that all the studied nanopowders in the dose of 10 mg/kg enhance the investigative activity of animals. Iron and titanium nanopowders cause a decrease in the distance travelled by the animals, which can be an evidence of the higher degree of reversible suppressing (negative) action on the central nervous system in this dose. In the dose of 500 mg/kg, only titanium nanopowder causes a substantial suppression of the motor activity of animals, which is exhibited as a decrease in all the parameters. Other studied nanopowders did not cause any pronounced effect on the motor and emotional behaviour of the animals.

<table>
<thead>
<tr>
<th></th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.6±1.7</td>
<td>4.6±0.8</td>
<td>5.8±1.0</td>
<td></td>
</tr>
<tr>
<td>14.1±1.5</td>
<td>9.3±1.2*</td>
<td>11.4±1.4*</td>
<td></td>
</tr>
<tr>
<td>18.4±2.6*</td>
<td>8.9±0.5*</td>
<td>9.6±0.5*</td>
<td></td>
</tr>
<tr>
<td>11.3±2.5</td>
<td>7.8±1.2*</td>
<td>9.4±1.4</td>
<td></td>
</tr>
<tr>
<td>13±2.7</td>
<td>7.5±0.6*</td>
<td>9.9±0.7*</td>
<td></td>
</tr>
<tr>
<td>25.6±2.2</td>
<td>44</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>18.6±2*</td>
<td>28</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>14.2±1.8*</td>
<td>28</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>8.33±3*</td>
<td>35</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

E — immobile time, s; F — number of vertical stands,
Macroscopic investigation of internal organs did not reveal toxic action.

All the nanopowders in the studied doses do not cause pathological effect on body temperature and mass; they also do not cause toxic action on internal organs.

On the basis of results obtained, we concluded that titanium, iron, aluminium oxide nanopowders obtained by means of electric explosion of metal wire relate to the 3rd (moderately toxic) class of danger. Copper nanopowder was related to the 2nd (toxic) class of danger.

Because nanopowders caused substantial suppression of the motor activity of the animals, we carried out a separate series of experiments (Table 2) in which the studied substances were introduced intragastrically once in the dose of 10 and 500 mg/kg. After the introduction, 1 h later, the animals were placed in the centre of the photosensor set-up TruScan (Coulbourn Instruments, USA) where the parameters of vertical and horizontal activity of mice were registered.

It was established that all the studied agents in the dose of 10 mg/kg increase the investigative reaction of animals (see Table 2, parameters H and I); iron and titanium nanopowders decrease the travelled distance, which can be an evidence of the decrease in motor activity.

In the dose of 500 mg/kg, only titanium nanopowder substantially suppresses the motor activity of the animals, which is expressed as a decrease in all the parameters. Other substances do not cause pronounced effect on the motor and emotional state of animals (see Table 2).

The introduction of iron and titanium nanopowders caused toxic effect on the CNS in the in vivo investigations, so the cytotoxic and stimulating action on primary and long-term cultivated human cells was studied in vitro.

It follows from the data obtained that the presence of iron nanopowder caused a weaker cytotoxic effect on primary endotheliocytes extracted from the umbilical vain of newborn babies, primary fibroblasts from the human gum tissue, the cells of cervical adenocarcinoma HeLa and the cells of breast cancer MCF-7; the effect was revealed at the concentrations of nanomaterials close to the maximal ones (100 µg/mL). Interesting properties of titanium nanopowder were discovered: it did not cause the death of cancer cells but turned out to be very toxic for primary cells even in the dose of 80 mg/mL.

Investigation of the effect of metal nanopowders on the secretion of anti-inflammatory cytokines showed that neither of the studied nanomaterials affects the concentration of anti-inflammatory cytokine IL-6 in the culture of primary cells.

The data obtained on the toxic action of titanium nanopowder on living systems confirm the fact that the same nanomaterials can cause ambiguous action both on an organism in general and on separate cells.

**Determination of acute toxicity of carbon nanomaterials**

Acute toxicity of carbon nanomaterials was determined after intragastric introduction in mice in the doses of 1000, 2000, 2500, 3000, 4000 and 5000 mg/kg.

In the recent years, the works appeared in which the broad possibilities to use carbon nanomaterials in biomedicine were demonstrated. First of all, this is the development of biosensors based on enzymatic electrodes fixed on carbon nanotubes, for the analysis of biological media (glucose, cholesterol, urea, amino acids etc.) [10, 11]. Second, the use of carbon nanotubes for intracellular therapy, that is, to deliver the necessary agent into the cell to cure such diseases as cancer, AIDS etc. [12, 13]. Third, the use of carbon nanotubes and carbon nanofibres directly to eliminate bacteria, microbes and even cancer cells [13].

Functionalization of carbon nanomaterials (N, B, F) is a promising direction in obtaining carbon tubes or fibres with new chemical properties. For example, the introduction of nitrogen into N-CNF allows one to change the electric conductance of carbon nanofibres [7]. On the other hand, the presence of ready nitrogen-containing functional groups on the surface of the carbon material may simplify immobilization of the target biological molecules for subsequent addressed delivery of a reagent into the cell. In this connection, investigation of the acute toxicity of carbon nanomaterials and nitrogen-functionalized carbon nanomaterials appears to be very urgent.
In this paper, we present the data on the toxic properties of carbon nanomaterials depending on the structure (graphite-like or amorphous), morphology (tubes, fibres), texture (specific surface), presence of nitrogen in the materials and its amount (0–17 mass % N), amount of metal catalyst remaining in the carbon material after washing (ash content 0–9 mass %). Results of investigations are presented in Table 3.

**CONCLUSIONS**

On the basis of investigations, the following conclusions were drawn:

1. Physicochemical characteristics of carbon nanofibres and the introduction of nitrogen into CNF have no effect on the average lethal dose; the whole group of the substances relates to the class 4 (low toxicity).

2. Studied carbon nanotubes (CNT) relate to the class 3 (moderately toxic) of compounds, are insoluble in water, which is their advantage over water-soluble carbon nanotubes. It is known that water-soluble carbon nanotubes, depending on their introduction into an animal organism, exhibit different toxic effects on living systems. In particular, after peroral introduction they get distributed over the tissues and organs excluding the brain, decrease the viability of cells; increase the content of the inflammatory marker interleukin-8. These nanotubes are also characterized by the high affinity to DNA molecule, which makes them potential mutagens. The main reason of the destroying action of carbon nanostructures is the induction of active oxygen forms and oxidation of biological molecules [14–16].

3. The toxicity of amorphous microporous carbon depends on nitrogen content of the material and decreases from the class 3 of moderately toxic substances (0 % N) to the class 4 (low toxicity) of substances (5 or 17 % N).

4. Low toxicity of the studied carbon nanomaterials determines their promising character for the development of nanostructured forms of medical preparations.

**REFERENCES**