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M.M. Zahornyi [1,3]***,***
O.P. Yavorovsky [2]***,***
V.M. Riabovol [2*]***,***
N.I. Tyschenko [1,3]***,***
T.F. Lobunets [1,3]***,***
T.V. Tomila [1]***,***
O.V. Shirokov [1]***,***
A.V. Ragulya [1,3]***,***
Ye.M. Anisimov [2]

MORPHOLOGICAL, SPECTRAL AND TOXICOLOGICAL FEATURES OF NEW COMPOSITE MATERIAL OF TITANIUM NANODIOXIDE WITH NANOSILVER FOR USE IN MEDICINE AND BIOLOGY

Frantsevich Institute for Problems of Materials Science, NAS of Ukraine [1]
Krzhizhanovsky str., 3, Kyiv, 03142, Ukraine
Bogomolets National Medical University [2]
T. Shevchenko blvd., 13, Kyiv, 01601, Ukraine
LLC «Nanotechcenter» [3]
Krzhizhanovsky str., 3, Kyiv, 03142, Ukraine
Інститут проблем матеріалознавства імені І.М. Францевича [1]
вул. Кржижановського, 3, Київ, 03142, Україна
Національний медичний університет імені О.О. Богомольця [2]
бул. Т. Шевченка, 13, Київ, 01601, Україна
ТОВ «Нанотехцентр» [3]
вул. Кржижановського, 3, Київ, 03142, Україна
[*]*e-mail: riabovol1@ukr.net*

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Ключові слова: *наноструктури, поверхневі дефекти, композит нанодіоксиду титану з наносріблом, нанопорошок діоксиду титану, гостра внутрішньоочеревинна токсичність, сенсибілізаційна дія, біологічна активність, анатаз*
Ключевые слова: *наноструктуры, поверхностные дефекты, композит нанодиоксида титана с наносеребром, нанопорошок диоксида титана, острая внутрибрюшинная токсичность, сенсибилизирующее действие, биологическая активность, анатаз*

Abstract. Morphological, spectral and toxicological features of new composite material of titanium nanodioxide with nanosilver for use in medicine and biology. Zahornyi M.M., Yavorovsky O.P., Riabovol V.M., Tyschenko N.I., Lobunets T.F., Tomila T.V., Shirokov O.V., Ragulya A.V., Anisimov Ye.M. *The results of this study indicate that titanium dioxide nanoparticles (nano-TiO_2) possess adsorptive, photocatalytic, bactericidal, virucidal and fungicidal properties, which are used in antibacterial coating, for air and water disinfection. In parallel with studies of the physicochemical characteristics of titanium dioxide, its toxicological assessment was carried out to prevent possible harmful effects on humans and the biosphere objects, followed by an assessment of the nano-TiO_2 hazard class. To enhance these useful properties of nano-TiO_2, nanopowders of titanium dioxide and a composite of titanium dioxide were synthesized with a silver (nano-TiO_2/Ag) by way of chemical precipitation of metatitanic acid adding silver nitrate to the composite at 500-600°C. It was stated that the synthesized nanostructures have the following characteristics: anatase crystal structure of TiO_2 (anatase, rutile, brookite – natural crystalline modifications of TiO_2), the size of Ag nanoparticles is 35-40 nm, TiO_2 – 13-20 nm. Nanocomposite has surface defects of the crystal lattice (oxygen vacancies, impurities, excess electrons or holes), silver nanoparticles are localized on the surface of anatase TiO_2, which increases adsorptive, photocatalytic, biological and specifically antibacterial properties of the composite material nano-TiO_2/Ag. According to the parameters of acute intraperitoneal toxicity, the studied nanocomposite anatase nano-TiO_2/Ag was classified as a moderately dangerous substance (material). Nano-TiO_2 and TiO_2/Ag nanocomposites do not cause local irritation to the skin, yet have a mildly irritating effect on the mucous membrane of the eye, and are also characterized by a weak sensitization effect.*

Реферат. Морфологические, спектральные и токсикологические особенности нового композитного материала нанодиоксида титана с наносеребром для использования в медицине и биологии. Загорный М.Н., Яворовский А.П., Рябовол В.Н., Тищенко Н.И., Лобунец Т.Ф., Томила Т.В., Широков А.В., Рагуля А.В., Анисимов Е.Н. *Сегодня актуальным является получение и использование диоксида титана (нано-TiO_2) с адсорбционными, бактерицидными, вирулицидными и фунгицидными свойствами для изготовления антибактериальных покрытий, для обеззараживания воздуха и воды. Параллельно с исследованиями физико-химических характеристик диоксида титана осуществлялась его токсикологическая оценка для предупреждения возможного вредного воздействия на человека и объекты биосферы с последующей оценкой класса опасности TiO_2. Для получения указанных полезных свойств нано-TiO_2 синтезировали нанопорошки диоксида титана и композит диоксида титана с серебром (нано-TiO_2/Ag) химическим осаждением метатитановой кислоты, с добавлением нитрата серебра для композита при температуре 500-600°С. Установлено, что синтезированные наноструктуры имеют следующие характеристики: анатазную кристаллическую структуру TiO_2 (анатаз, рутил, брукит – природные кристаллические модификации TiO_2), размер наночастиц Ag составляет 35-40 нм, TiO_2 – 13-20 нм. Нанокомпозит имеет поверхностные дефекты кристаллической решетки (вакансии кислорода, примеси, избыточные электроны или дырки), также на поверхности анатаза TiO_2 локализованы наночастицы серебра, что повышает адсорбционную, фотокаталитическую, биологическую (особенно антибактериальную) активность композитного материала нано-TiO_2/Ag. По параметрам острой внутрибрюшинной токсичности исследованный нанокомпозит отнесен к умеренно опасным веществам (материалам). Нано-TiO_2 и нанокомпозит TiO_2/Ag не оказывают местно-раздражающего действия на кожу, но имеют слабо раздражающее действие на слизистую оболочку глаза, также характеризуются слабо выраженной способностью к сенсибилизации.*

Nanomaterials obtained on the basis of nanoparticles of metals and their compounds are increasingly used in various fields of economic activity, biology and medicine. In Ukraine and the world, special attention is paid to the synthesis of nanoparticles based on titanium dioxide (TiO_2). It exists in nature in three crystal modifications - anatase, rutile, brookite.

The peculiarity of titanium dioxide nanoparticles is that they have high photocatalytic activity under the action of ultraviolet radiation. As a result of experimental studies (Y.B. Pankivska et al. [5]) it was established that due to high photocatalytic activity nano-TiO_2 can be used as an antimicrobial agent with high bactericidal, virucidal and fungicidal effect. This becomes especially relevant in the era of increased resistance of microorganisms to the action of antibiotics and disinfectants.

In addition, an attempt was made to use nanotitanium dioxide for the manufacture of antibacterial coatings, impregnation of filters with nanomaterials for disinfection of water, air in closed rooms, etc. [5, 12, 16].

The search for new materials with increased photocatalytic properties due to the addition of metals and metal oxide compounds with previously known biocidal properties (silver, copper oxide, iron oxide, zinc oxide, etc.) to titanium dioxide is being done. One of these newly created nanomaterials was a composite based on nanotitanium dioxide and nanosilver, obtained at I.M. Frantsevich Institute for Problems of Materials Science, NAS of Ukraine

However, it is well known that a higher physicochemical activity of nanoparticles is accompanied by an increase in their biological activity, and different compositions of nano-TiO_2 can have different toxicity depending on the crystal structure, size, surface characteristics, particle shape and surface area. In addition, the authors' research indicates that TiO_2 nanoparticles, having entered the cell by endocytosis, are not absorbed by organelles and do not undergo biodegradation inside the cell. Researchers believe that nano-TiO_2 causes a nonspecific increase in the concentration of intracellular reactive oxygen in the cell, which leads to cell apoptosis and/or the initiation of an inflammatory reaction. When studying the cytotoxicity of nano-TiO_2 for human lung epithelial cells, it was established that the crystalline form of anatase is more toxic than a mixture of anatase and rutile [11].

This indicates that, in parallel with the studies of physical and chemical characteristics, it is necessary to carry out their toxicological assessment to prevent possible harmful effects on humans and objects of the biosphere.

The object of our research was the above-mentioned newly created composite material of nanotitanium dioxide, modified with nanosilver, developed at I.M. Frantsevich Institute for Problems of Materials Science NAS of Ukraine for use in biology and medicine.

The purpose of the research was to study the morphological features of the TiO_2/Ag nanocomposite structure, to study the optical and toxicological properties of this material.

MATERIALS AND METHODS OF RESEARCH

Nanopowders of titanium dioxide and a composite of titanium dioxide with silver (nano-TiO_2/Ag) were synthesized by chemical precipitation of metatitanic acid, with the addition of silver nitrate in the amount of 4% of the mass of the composite at a temperature of 500-600°C.

Morphological, structural, spectral and optical properties of nano-TiO_2 and nano-TiO_2/Ag objects were characterized using transmission electron microscopy (TEM), X-ray phase analysis (XRPA), infrared spectroscopy (IR) and laser granulometry. The study of the porous structure of these nano-powders was carried out by the method of determining the specific surface area (express method of thermal desorption of nitrogen) on the HKh-1 device according to the methodology [2].

TEM studies of the samples were carried out with the help of a JEM-1400 electron microscope (JEOL, Japan) at instrumental magnification from 2000 to 100000 at an accelerating voltage of 80 kV according to the method of work [13].

X-ray diffraction of synthesized TiO_2, TiO_2/Ag samples was studied using a DRON-3M X-ray diffractometer according to the method [14].

IR spectroscopic studies of the samples were carried out on a FSM-1201 Fourier spectrometer in the wavelength range of 4000-400 cm^{-1}. For measurements, the studied samples were thoroughly mixed with KBr powder in a ratio (1:300 mg) and the resulting mixture was pressed into transparent tablets with a diameter of 13 mm [3].

Since TiO_2 and TiO_2/Ag nanoparticles showed a tendency to agglomerate and were unstable in aqueous suspensions, we searched for chemical compounds that could act as a factor in stabilizing suspensions (saline solution, solutions of sodium citrate, glucose, etc.), which is important for planning and conducting toxicological experiments, in particular, dosing of nanopowders when administered per os, intraperitoneally, intranasally, etc. The size of nanoparticle agglomerates in the dispersion medium was determined by laser granulometry using the Analysette 12 DynaSizer device [8].

The local irritant effect of TiO_2 and TiO_2/Ag nanopowders was studied by applying an ointment obtained on the basis of petroleum jelly and nanomaterial when mixed in a ratio of 1:1 by weight on the right side of pre-depilated skin (5x5 cm on symmetrical areas of the back, the left side for control) of guinea pigs (the control and research groups consisted of 4 animals each). The degree of erythema and the extent of skin edema were assessed. The difference in skin irritation between experimental and control groups was compared [4].

The study of the local effect of nanopowders on the mucous membrane of the eye was carried out by injecting 10 mg of native nanopowder TiO_2 and TiO_2/Ag into the conjunctival sac of the right eye (the left control eye) of a rabbit (the studied groups consisted of 3 animals each). Irritation of the mucous membrane of the eye was evaluated according to the degree of hyperemia, edema, secretions from the mucous membrane [4].

The acute toxicity of TiO_2 and TiO_2/Ag nanomaterials in rats was studied by intraperitoneal injection of a suspension in physiological solution (0.9% NaCl) in doses from 1,000 mg/kg to 13,000 mg/kg (13 laboratory animals). The duration of observation of the condition of the animals after administration was 14 days, the manifestations of signs of intoxication and lethality were assessed. Rats were removed from the experiment by the introduction of 1% propofol. A general blood test was performed [4]. The acute toxicity of nano-TiO_2 and nano-Ag/TiO_2 was studied in mice at doses of 4,000 mg/kg, 7,000 mg/kg, and 10,000 mg/kg (control and research groups of 6 animals each) by intraperitoneal administration of suspension in physiological solution (0.9% NaCl). Observations and conclusions from the experiment were carried out under similar conditions as in the experiment on rats [4].

The sensitizing effect of nano-TiO_2 and nano-Ag/TiO_2 was studied on guinea pigs (control and research groups of 4 animals each) by intradermal injection of suspensions of nanopowders at a dose of 200 μg into the ear of the guinea pig [4].

The conditions for keeping and using laboratory animals corresponded to the ethical rules and provisions of the "European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes" (Strasbourg, 1986).

The effect of the composite on the functional activity of donor mononuclear cells was studied in vitro. After informed consent, peripheral blood was taken from 30 donors (almost healthy people) for in vitro research. Mononuclear cells were isolated from peripheral blood and cultivated on RPMI-1640 medium, then the cell suspension was incubated for 24 hours in a CO_2 incubator at 37°C without a stimulating agent, under stimulation with the PHA mitogen and nanomaterials in doses of 30 μg/ml. The concentration of cytokines (IL-1,IL-4) [7] was studied by ELISA method in supernates of mononuclear cells. The research was conducted in compliance with the principles of bioethics set forth in the Helsinki Declaration "Ethical Principles of Medical Research Involving Humans" and agreed by the Commission on Bioethical Expertise and Research Ethics of the O.O. National Medical University Bogomoltsia No. 128 of 12/23/2019.

Non-parametric criteria were used for statistical processing: Wilcoxon's W-test, Kruskal-Wallis rank univariate analysis, multiple comparisons according to Dunn's test. The difference was considered statistically significant at $p<0.05$. Statistical processing of information was carried out using the MedStat v.5.2 software package (Copyright © 2003-2019) [1].

RESULTS AND DISCUSSION

The morphology of TiO_2 nanopowders was analyzed using transmission electron microscopy (TEM). Figure 1 shows an electron microscopic image of TiO_2 nanoparticles (Institute for Problems of Materials Science – IPMS) and TiO_2-P25 (a commercial sample of titanium dioxide, namely TiO_2-P25 produced by Evonik Industries AG, Germany, was chosen as a comparative sample). It was found that TiO_2 nanopowder (IPMS) contains soft aggregates, nanoparticles mainly 20-30 nm in size (Fig. 1a). TiO_2 nanopowder (IPMS) has a developed surface structure due to the presence of mesopores (pores 2-50 nm) and a specific surface area of 50.84 m^2/g. Nanoparticles of TiO_2-P25 have mainly a square shape with a size of 20-25 nm (Fig. 1b).

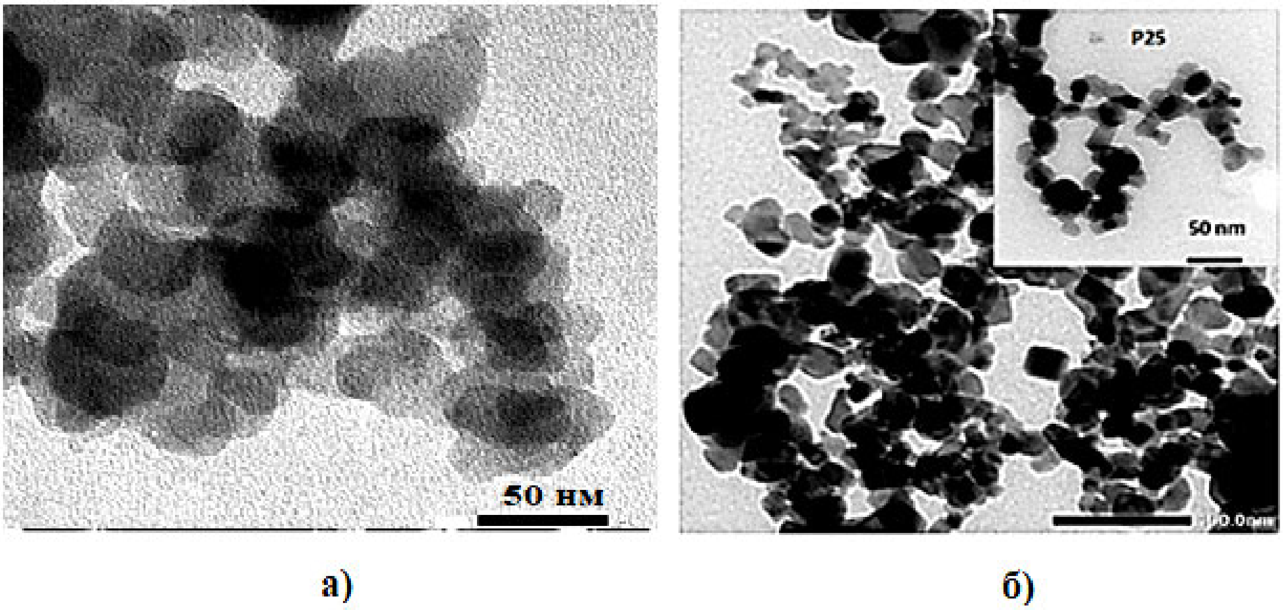

Fig. 1. Surface morphology of TiO_2 nanopowders
a) – transmission microscopy image of the TiO_2 surface,
b) – transmission microscopy image of the TiO_2-P25 surface

Surface structural defects of TiO_2 (formation of non-stoichiometric areas on the surface of anatase) can act as active centers, and also provide the possibility of additional chemical modification of the surface of the oxide with metal (nano-Ag) for purposeful regulation of the TiO_2 structure and carrying out catalytic reactions, increasing biological activity, in particular biocidal action.

TEM images of the surface of the TiO_2/Ag nanocomposite sample confirm the deposition of silver on the oxide surface (Fig. 2). We observe "ball-shaped" silver particles (red area), while Ag particles have an average size of 35-40 nm, and TiO_2 particles – 13-20 nm. The specific surface area of nano-TiO_2/Ag is 50.11 m^2/g.

Stabilization of the suspension of nanopowders with a glucose-citrate buffer (4 g of glucose, 1 g of sodium citrate, 100 ml of distilled water) contributed to a significant reduction of agglomerated complexes from the size of agglomerates of 300-500 nm to 40-50 nm [8].

We used X-ray phase analysis (XRPA) to identify the type of TiO_2 crystalline phase. The diffractogram [3] is used to study the structure of titanium dioxide and other objects (crystalline, amorphous-crystalline). In addition, the authors Der-Shing Lee and Yu-Wen Chen [10] also did not observe the formation of other phases except TiO_2 of anatase modification in TiO_2 samples in the range of silver concentrations of 2-3.8 wt.%.

The decrease in the size of TiO_2 particles when Ti^{4+} ions are replaced by Ag^+ ions can be associated with the deposition of silver on the TiO_2 surface, which leads to a disorder of structural symmetry (in agreement with IR spectroscopy), and therefore to reduction of the size of nanoparticles [3]. We proved this with the help of TEM – research of samples (Fig. 2).

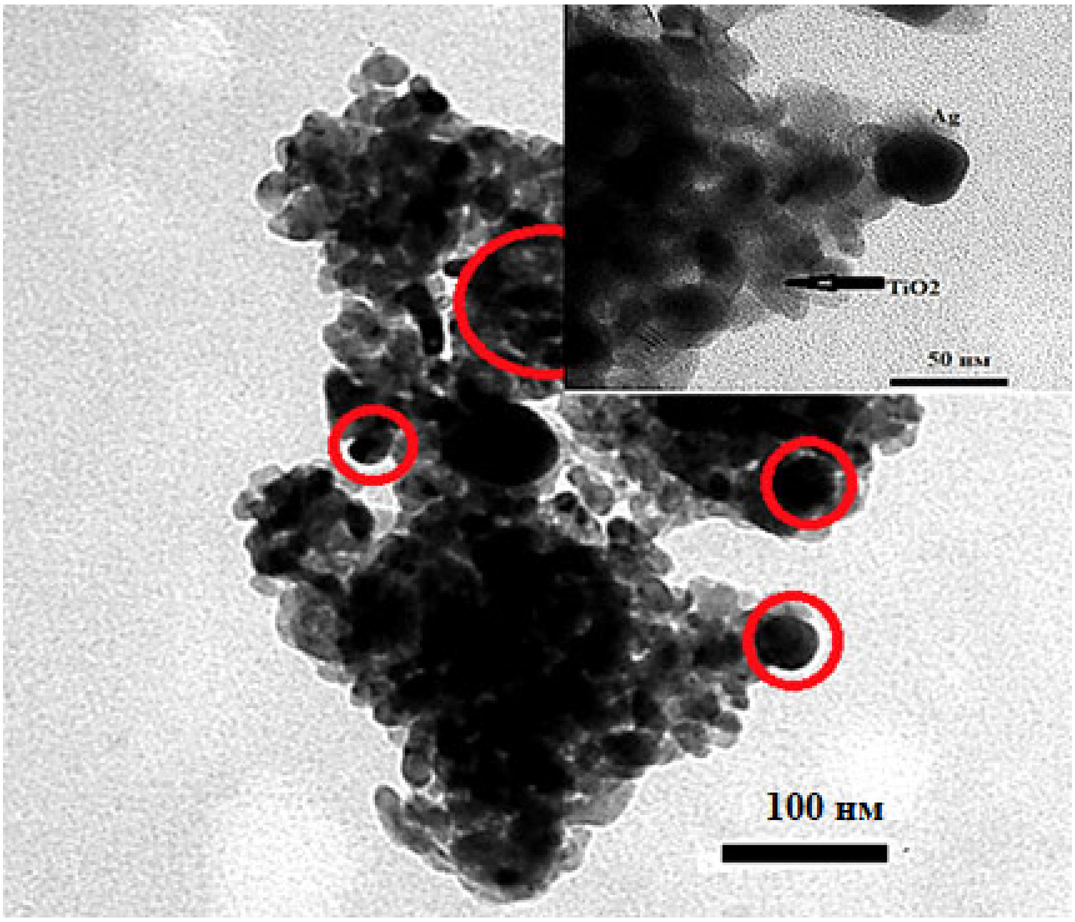

Fig. 2. Morphology of TiO_2 nanocomposite particles with 4 wt.% Ag (transmission electronic microscopy)

Analysis of the IR spectra [3] of the studied samples showed that the sample (nano-TiO_2 anatase) is characterized by bands of absorption of Ti-O oscillations with frequencies ν~ 504 and 664 cm^{-1}. For the IR spectrum of the other sample (nano-TiO_2 anatase + 4 wt.% Ag) there is a shift of absorption frequencies of Ti-O oscillations in the long-wave direction. Frequencies ν ~ 1110 and 1188 cm^{-1} characterize surface vibrations of functional groups (–OH) and are associated with the modification method. Spectral studies prove the formation of only a crystalline anatase modification with hydroxyl groups and a silver phase (concordance with XRPA [3]), which can serve as a basis for recommendations on the use of a composite material as an adsorbent. Hydroxyl groups which are present on the defective surface of TiO_2 become quite active as a result of capturing a hole with subsequent formation of •OH and other active forms of oxygen (ion radicals •OH, $O_2^{\bullet-}$, O^-), which leads to an increase in adsorption capacity and biological activity of anatase [5, 15]. In addition to oxygen vacancies, other surface defects may also be present for anatase depending on the synthesis method, the chemical composition of the air in which the powder is stored, and the storage conditions (darkness or light).

Our toxicological studies have shown that TiO_2 and TiO_2/Ag nanopowders (ointment based on petroleum jelly 1:1) do not have a local irritant effect on the skin of guinea pigs. TiO_2 and TiO_2/Ag nanopowders in a dose of 10 mg show a mildly irritating effect on the mucous membrane of the rabbit's eye.

By intradermal injection of suspensions of nanopowders in a dose of 200 μg into the ear of a guinea pig, it was established that nano-TiO_2 and nano-TiO_2/Ag can cause a weakly expressed sensitization effect.

Studies of acute toxicity by intraperitoneal administration to mice revealed that a dose of 10,000 mg/kg TiO_2 and TiO_2/Ag of nanopowders causes their 100% death. A dose of 7,000 mg/kg of nano-TiO_2 caused partial death of mice, but it was absolutely lethal for nano-TiO_2/Ag. A dose of 4,000 mg/kg did not lead to the death of mice when nano-TiO_2 was administered, but it caused their partial death when nano-TiO_2-Ag was administered. That is, TiO_2 nanopowder turned out to be relatively less toxic compared to TiO_2/Ag.

In contrast to mice, intraperitoneal administration of doses of the studied nanopowders from 1,000 mg/kg to 13,000 mg/kg to rats did not cause their death, but led to signs of general intoxication (depression, refusal of feed), as well as to a decrease in the content of erythrocytes and platelets in the peripheral blood of experimental animals.

At determined lethal doses (from 4 to 10,000 mg/kg of test substances administered to mice), TiO_2 nanopowders are preliminarily in accordance with Ukrainian State Standard 12.1.007-76 "System of labor safety standards. Hazardous substances. Classification and general safety requirements" can be attributed to the 4th class (less dangerous), and the TiO_2/Ag nanocomposite – to the 3rd class (moderately dangerous) of hazardous substances.

Cytokines regulate the effects of the immune system. Experiments on the study of the effect of nanomaterials on the immune system, conducted in vitro, showed that the nanomaterial in concentrations of 30 μg/ml nano-TiO_2-Ag (the dose corresponding to the possible entry into the body of the operator of nanoproduction by inhalation) is able to increase the functional activity of mononuclear cells of peripheral blood by the production of the pro-inflammatory cytokine IL-1 (interleukin 1 is produced mainly by macrophages in response to damage to the body, increases the production of other pro-inflammatory cytokines) and IL-4 production (interleukin 4 activates B-lymphocytes, switches IgM production to IgG4 or IgE production and activates T-lymphocytes involved in the allergic reaction of the cell type) in donors ($p<0.05$) (Fig. 3 -4).

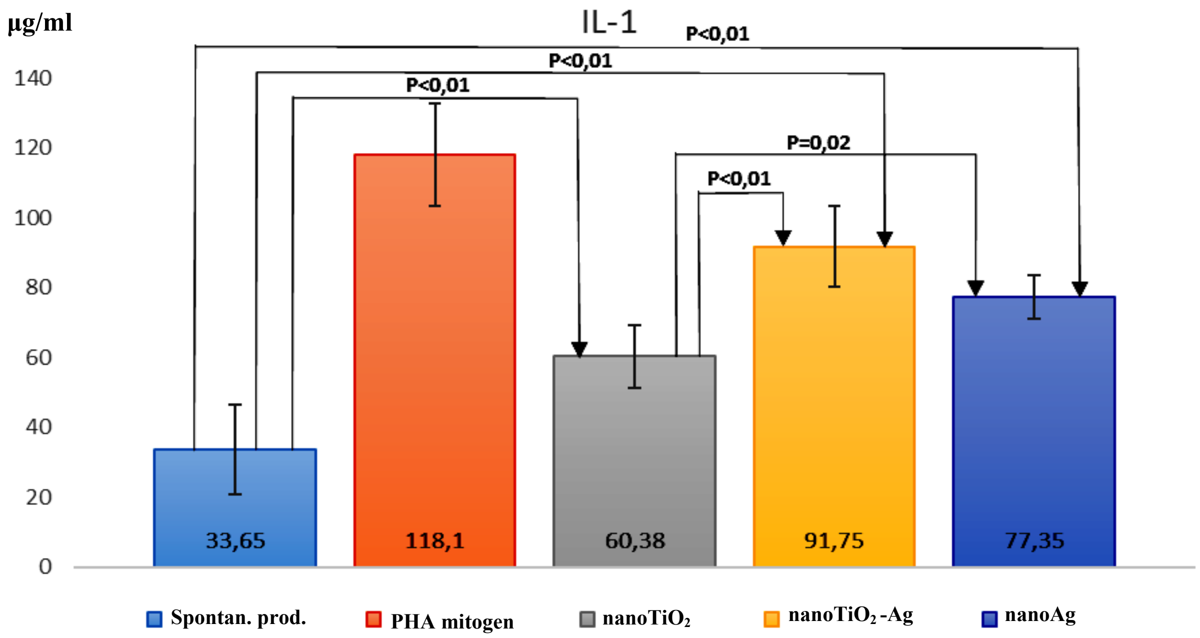

Fig. 3. Comparative production of IL-1 by mononuclear cells *in vitro* in donors under the influence of nanomaterials

The above results of *in vitro* research indicate the potential possibility of TiO_2-Ag nanocomposite to form chronic inflammation and allergic reactions in the category of workers who are exposed to the mentioned nanopowders in production conditions.

Thus, our toxicological studies of newly synthesized TiO_2 and TiO_2/Ag nanopowders confirm the results of the previously conducted toxicological and hygienic evaluation of nanopowders of titanium nitride, chromium disilicide, barium titanate, and zirconium dioxide, synthesized at I.M. Frantsevich Institute for Problems of Materials Science NAS of Ukraine

Thus, nanopowders of metals, their oxygenated and oxygen-free compounds are less toxic and dangerous when entering the body through the gastrointestinal tract and skin, but exhibit a more pronounced toxic effect when entering the body by inhalation [6, 7]. All of the nanoparticles tested by us do not irritate the skin, although they can have a mildly irritating effect when they come into contact with the mucous membrane of the eye. When nanopowders enter the living body, the immune system reacts, forming toxic-allergic reactions in response.

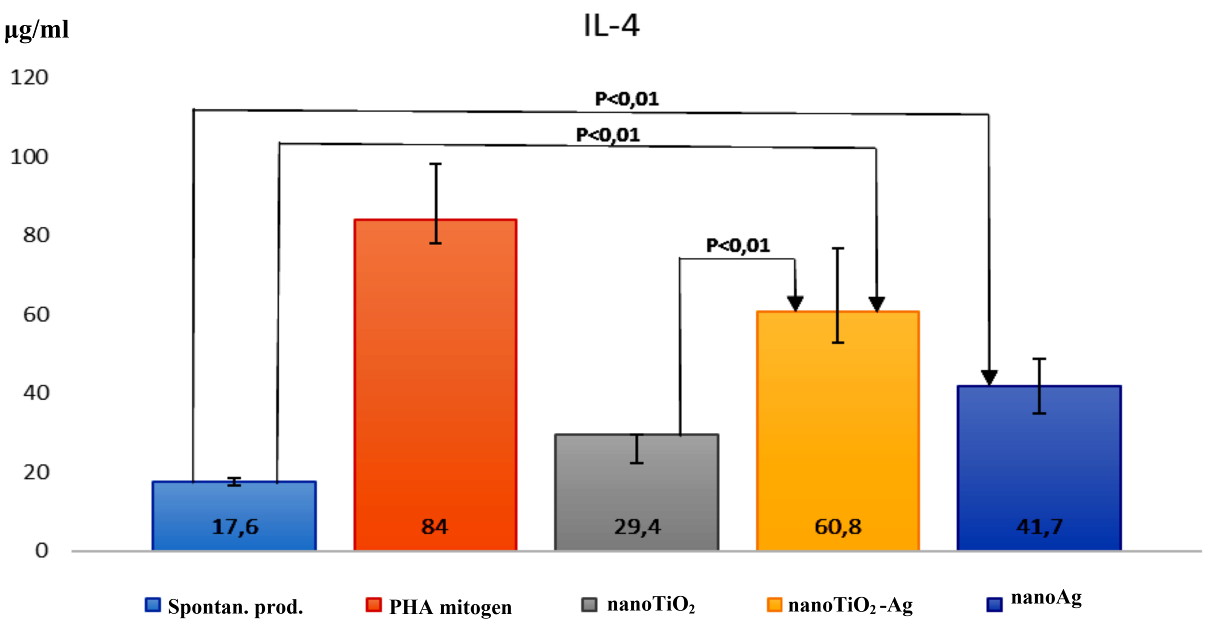

Fig. 4. Comparative production of IL-1 by mononuclear cells *in vitro* in donors under the influence of nanomaterials

Regarding a more pronounced toxic effect of nano- TiO_2/Ag compared to pure nano-TiO_2, these data are fully consistent with the research of the Institute of Microbiology and Virology named after D.K. Zabolotny, National Academy of Sciences of Ukraine, carried out on MDBK, MDCK, HepG2 cell cultures, which showed more pronounced biocidal properties of the TiO_2+Ag nanocomposite precisely at a concentration of 4% silver [9, 10]. The nano-TiO_2/Ag composite material with a silver content not exceeding 4% exhibits a more pronounced biocidal effect (virulicidal, bactericidal, fungicidal), but does not destroy the living cell itself.

CONCLUSIONS

1. TiO2/Ag nanopowder, synthesized by the chemical deposition method, with a concentration of silver on the surface of 4 wt.% has its own physical and chemical features, which concern to the morphology of nanoparticles, the molecular structure of their surface, and toxicological properties.
2. Using the method of transmission electron microscopy, it was established that Ag nanoparticles have an average size of the dispersed Ag phase of 35-40 nm, and TiO_2 – 13-20 nm in the composite material. Nanosilver particles are localized on the surface of nanotitanium dioxide. TiO_2 and TiO_2/Ag nanopowders have a developed surface structure with mesopores. Laser granulometry showed that ultrasonic dispersing in glucose-citrate buffer can significantly reduce the size of agglomerates in TiO_2 and TiO_2/Ag nanopowders.
3. Spectral analysis (XRPA, IR) confirmed the purity of anatase modification of TiO_2 and TiO_2/Ag nanopowders. Defects detected on the surface of synthesized nano-TiO_2 can act as active centers that make it possible to modify the bactericidal properties of metal (Ag) with nanoparticles to improve antimicrobial, photocatalytic, and biocompatible properties.
4. Nano-TiO_2 and nanocomposite TiO_2/Ag do not have a local irritating effect on the skin, have a weak irritating effect on the mucous membrane of the eye and are characterized by weakly expressed sensitizing properties. Potentially, nano-TiO_2-Ag can cause allergic reactions and chronic inflammation in workers.
5. It is necessary to further study the toxicological properties of nano-TiO_2/Ag in order to find out the mechanisms of their biological action at the cellular and molecular genetic levels, to study possible manifestations of remote effects for the justification of hygienic standards in the air of the workplace zones.

Contributors:

Zahornyi M.M. – writing – original draft, management;

Yavorovsky O.P. – project administration, conceptualization, methodology, writing – review and editing;

Riabovol V.M. – methodology, research, formal analysis;

Tyschenko N.I. – research, resources;

Lobunets T.F., Tomila T.V. – research;

Shirokov O.V. – formal analysis;

Ragulya A.V. – project administration, finding financial support;

Anisimov Ye.M. – research, formal analysis.

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Conflict of interests. The authors declare no conflict of interest.

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