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Olha Smirnova
Department of technical electrochemistry
National Technical University "Kharkiv Polytechnic Institute"
Kharkiv, Ukraine
oleleo1970@gmail.com

Oleksij Pylypenko
Department of Chemistry and Integrated Technologies
O. M. Beketov National University of Urban Economy in Kharkiv
Kharkiv, Ukraine
opilipenko1984@gmail.com

Aleksandr Brovin
Department of technical electrochemistry
National Technical University "Kharkiv Polytechnic Institute"
Kharkiv, Ukraine
postbox.brovin@gmail.com

Andrej Nikonov
Department of Dentistry
V. N. Karazin Kharkiv National University
Kharkiv, Ukraine
a.ju.nikonov@gmail.com

Svitlana Hordiienko
Department of Dentistry
V. N. Karazin Kharkiv National University
Kharkiv, Ukraine
svetlana_200969@ukr.net

Valerija Rusanova
Department of Orthopedic Dentistry
Kharkiv Medical Academy of Postgraduate Educat
Kharkiv, Ukraine
doctorvaleria11@gmail.com

***Abstract*—The opportunities of the reducing synthesis were studied to synthesize gold nanoparticles in a wide range of their sizes. For this purpose, different well-known (classic) and innovative reducing agents have been used. Based on the obtained colloids, the preparations were prepared that were used for medical *in vitro* and *in vivo* studies. The experiment showed a positive outcome especially for the particles of smaller sizes (~ 6 nm). These particles have a high potential for the successful use in medicine due to their high biological activity. The investigation showed the unavailability of an essential negative effect of gold nanoparticles on internal organs of experimental animals.**

Keywords—synthesis, chemical reaction, gold compound, reducing agent, stabilizer, colloidal solution (sol), gold nanoparticle, and biological activity.

I. Introduction

The application of gold by human society has a centuries -old history starting from Ancient Egypt, India, China and up to the present [1, 2]. In the middle Ages, Paracelsus obtained the colloidal gold through the reduction of gold salts by alcoholic and oily plant extracts. Starting from the XVII century the reaction with the use of tin chloride (II) as a reducing agent came to light. In the middle of the XIX century *M. Faraday* used white phosphorus for the CS_2 solution as a reducing agent for the synthesis of colloidal gold.

At the end of the XIX century and in the early 20th century *R. Zsigmondy* continued the studies of the colloidal gold solutions. He carried out the synthesis using different reducing agents, in particular hydrogen peroxide, formaldehyde, ethanol and white phosphorus. In the XX century, *T. Svedberg* carried out synthesis of colloidal gold using 25 reagents and among them were inorganic and organic substances that possessed reducing properties [4].

At the turn of XX and XXI centuries, the development of nanotechnologies gave the "second birth" to the colloidal gold. It can be explained by the fact that the unique physical and chemical properties of gold nanoparticles found wide application in the different fields of contemporary science, in particular, in nanotechnology and nanomedicine [5, 6]. The intensive studies of gold nanoparticles revealed the availability of many valuable properties in them and it gives grounds for the development of anticancer and antimicrobial preparations and the instruments for the drug delivery to the cells of protein molecules and DNA [7]. It contributes to the progress in the treatment of patients suffering from the diseases of a different etiology.

Colloidal gold finds a wide application for immunochemical studies. The fact that gold granules have hydrophobic properties and the electrostatic charge on their surface gave an impetus to the production of specific markers with a large number of macromolecules such as antibodies, pectins, ferments, different proteins, glycoproteids and polysaccharides [8].

The interaction of bacteria with colloidal gold particles can happen in a different way. It can be the adsorption of gold on the cell surface changing no size of the particles; the adsorption of gold with the simultaneous aggregation of particles, aggregation of sol particles with no noticeable adsorption on the cell surface [9]. The processes of adsorption, flocculation and recrystallization of colloidal gold can be related to the activities of respiratory chain and the generation of the transmembrane electrochemical potential on the cytoplasmic membrane of bacterial cells.

II. Problem Statement

An easy gold reduction can be explained by the highest electrochemical potential of it due to which gold cations act as strong oxidizers [10]. The reducing synthesis carried out to get gold nanoparticles is relating to the condensation methods of the synthesis of colloidal systems in which the nanoparticles of the reduced metal are formed from the ions of appropriate gold salts.

Today, the best known method used for the preparation of gold nanoparticles is the citrate method [11, 12]. It allows us to obtain spherical gold particles with the sizes of 15 to 150 nm. It is fair to say that the authors of this method are *J. Turkevich* и *G. Frens*. On average, the gold particles

obtained through the reduction of gold from $HAuCl_4$ solution by sodium citrate have the sizes of approximately 15 to 20 nm [13]. These are more uniform in their shape and size in comparison with the particles that were obtained using other reducing agents (white phosphorus, acetone, tannin, oxalic acid, hydroxylamine, carbon oxide (II), acetylene and citric acid). The authors showed that a change in the $HAuCl_4$ and sodium citrate ratio in the reaction mixture results in a change of the size of formed particles. However, in the course of the citrate synthesis of nanogold the intermediate products of the reaction are formed and these can be attributed to the drawbacks of the given method so far as medicinal drug is concerned.

Gold nanoparticles of a smaller size (8 to 10 nm) can be obtained when changing the reagent addition procedure. First, sodium citrate is added and then $HAuCl_4$. The nanoparticles of smaller sizes (3 to10 nm) cannot be obtained using the citrate method. To resolve this complicated problem the scientists used such reducing agents as white phosphorus, sodium and potassium thiocyanates, triphenyl phosphine, tannin acid, sodium borohydride and sodium cyanoborohydride. It should be noted that the problem of the use of sodium borohydride consists in a bad controllability of the processes of its hydrolysis and the infiltration of boron into the composition of metal nanoparticles.

The scientific paper [14] analyzes the citrate method of the chemical synthesis of colloidal gold and it also suggests the original dependences of the average volume of the particles on their concentration in the solution. The stability of gold nanoparticles was also studied and the transmission and absorption spectra were measured.

The scientific paper [4] describes the composition of gold nanoparticles and it gives the schematic diagram of the colloidal gold particle; the survey of the methods used for the synthesis and stabilization of nanoparticles was done and the examples of the biomedical application of colloidal gold were given, in particular as a carrier of antineoplastic agents.

Actually, the drawback of many syntheses is the toxicity of the used reducing agents (reagents). In this case, the main criterion for the application of the gold nanoparticle-based suspensions for medical purposes is the unavailability of their cytotoxicity *in vitro* and the toxicity at the level of the organism. To attain this goal, the reducing synthesis of gold nanoparticles should only be carried out under the condition of the use of absolutely safe chemical reagents with no formation of toxic intermediate and final reaction products. The obtained suspensions should totally be biocompatible with the human organism and suitable for therapeutic purposes.

In spite of the apparent progress in the studies of gold nanoparticles, the problems of their toxicity have not been solved completely yet. The issues relating to overdosing, minimum doses and possible effect on the organs and tissues are not disclosed, including many other problems [8]. The authors of [2] arrived to the conclusion that gold nanoparticles penetrate inside the cells, however, spherical gold nanoparticles in their pure state and in the combination with many substances have no toxic effect on the cells.

In this connection, the reducing synthesis with the use of amino acids is of great practical interest. The scientific paper [9] states that the gold-to-amino acid ratio less than 1:10 provides the reduction of gold to the metal with the formation of fine colloidal systems with the particle diameter less than 1 μm. The reduction of gold occurs the most rapidly and completely in the acid medium. All the amino acids can act as reducing agents. In addition to different organic compounds, sodium carbonate and iron hydroxide can act as the stabilizing agents of the colloidal particles of gold.

III. Objective and Research Tasks

The purpose of this study was to obtain preparations based on gold nanoparticles using reducing synthesis, to study their properties and prospects for use in medicine. This scientific paper delves into the solution of the following problems:

- Carrying out the reducing synthesis and preparing stable gold sols;
- Studying physical, chemical and biological properties of the gold-based nanosystems;
- Evaluation of the biological activity of the obtained preparations for reference strains of microorganisms;
- Comparative evaluation of the effect of gold nanoparticles and a standard antimycotic drug in the treatment of fungal infections caused by *Candida Albicans.*

IV. Methods of Experimental Investigations

To carry out chemical synthesis the following components were used: the distilled water, hydrogen tetrachloraurate (III) ($HAuCl_4 \cdot 4H_2O$), the reducing agents (hydrogen peroxide, sodium citrate, oxalic acid, sugar and glycine), and the stabilizer – sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$). All the reagents had a high purity degree.

First, we prepared the 0.001 M solution of hydrogen tetrachloraurate (III) by dissolving 0.412 g/dm^3 $HAuCl_4 \cdot 4H_2O$ in the 1000 ml of distilled water. Then, the obtained solution in the amount of 20 ml was poured into the chemically resistant beaker of 100 ml. The beaker was put on the electric stove, the solution was brought to the boiling point and it was boiled for 2 minutes. Afterwards, in order to prevent the aggregation of nanoparticles the beaker was put onto the magnetic mixer that was preliminary heated to 100 °C and the solution continued to boil at continuous mixing (360 rpm).

The reducing agent (~ 2 ml) was added using the glass pipette and the mixture was boiled for 10 minutes more starting from the moment of observed colored, chemical and structural transformations. To maintain a constant volume of the solution (~ 22 ml) we periodically added the doses of distilled water using the 5 ml pipette. Then, the beaker was taken off the magnetic mixer and the stabilizing agent was added and the gold sol was cooled at room temperature and it was held for additional 10 to 15 minutes to provide final ripening. The prepared colloid was poured into the sterile hermetical vessel and it was stored in a dark place at a temperature of 10 to 15 °C.

The sizes of gold nanoparticles were measured according to [15] using the device Zetasizer Nano ZS/ZSP/S. The microimages of nanoparticles were obtained at NTU "KhPI" using the transmission electron microscope PEM–100.

This scientific paper describes *in vitro* studies of the antimicrobial and antimycotic (antifungal) properties peculiar for gold nanoparticle-based medical preparations. For this purpose, the reference strains of microorganisms were prepared at the Laboratory of the I. Mechnikov Institute for Microbiology and Immunology of the National Academy of Medical Sciences of Ukraine. The strains of *Staphylococcus aureus* (ATCC 25923), *E.coli* (ATCC 25922), *Candida albicans* (ATCC 885/653) and also the pathogenic microflora association strains were used as the test-strains of the microorganisms. The nanogold content was the same for all the suspensions, in the amount of 25 μg/ml.

The sensitivity of drugs to *S.aureus, E.coli* and the mixed flora was defined using the method of "wells". After the incubation in Petri dishes with the bacterial seeding during 24 hours at a temperature of 37 ºC, the availability of the growth of microorganisms around the seed-spot with test substance was analyzed.

The sensitivity of *Candida albicans* was defined by means of the material seeding in Petri dishes that contained the nutritional medium (Sabouraud medium on feed water). After the incubation in the Petri dishes during 48 to 72 hours at a temperature of 37 ºC, the availability of the growth of microorganisms around the seed spot with the test substance was analyzed. The antibacterial activity was assessed according to the following criteria: insignificant effect in the range of 11 to 14 mm; moderate effect in the range of 15 to 19 mm and significant effect in the range of 20 to 40 mm.

The studies *in vivo* were carried out on the laboratory sexually mature rabbits with an average mass of 3.2 kg. For the comparative studies, the following preparations were used: 1) "Candid", a 1% solution for topical application, the active ingredient was clotrimazole, i.e. the antimycotic agent with the wide spectrum of effect; 2) gold nanoparticle – based agent, i.e. fine water suspension (the particle size of ~ 6 nm).

The rabbits were subdivided into the 4 groups, each group counted 10 rabbits. Group № 1 was the reference one, group № 2 was infected with *Candida Albicans* and was treated with the "Candid" preparation, group № 3 was infected with *Candida Albicans* and received treatment with the gold nanoparticle-based preparation and group № 4 was infected with *Candida Albicans* and received no treatment. The thiopental-anesthetized rabbits were taken out of the experiment on the 21 day of the investigation. After the experiment, the specimens of their internal organs (kidney, liver and heart) were ablated and placed into the 10 % formalin solution for the fixation and carried out their histological examination.

V. Research Data And Discussion

To obtain gold nanoparticles as a component of the colloids (sols) we carried out the chemical synthesis using different reducing agents. The choice of the reagents was based on the criteria of a minimum toxicity of original substances and end-products of their oxidation and biocompatibility with the human body. The classic methods of the reducing synthesis were used to carry out the experiment. The obtained experimental data are given in Fig. 1 and Fig. 2 and summarized in Table 1.

The obtained data are indicative of the fact that the size of gold nanoparticles depends on the type and the amount of the reducing agent. In the case of the use of hydrogen peroxide an increase in the amount of the reducing agent results in the aggregation of gold nanoparticles and in the change of their sizes in the range of 6 nm to 18 nm.

Fig. 1. The images of test objects: on the left we can see the solution of hydrogen tetrachloraurates (III) and in the center and on the right we can see colloidal gold solutions.

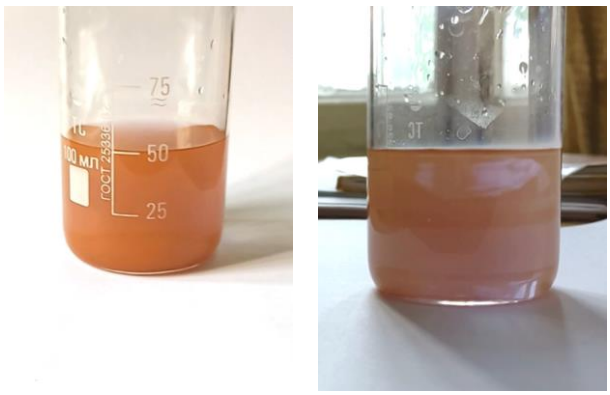

Fig. 2. The image of the gold sol that was obtained using hydrogen peroxide as a reducing agent.

TABLE I. Description of Gold Sols That Were Obtained Through Chemical Synthesis Using Different Reducing Agents

Type of reducing agent	***Ox* and *Red* ratio, mg : mg**	**Description of gold sols**		
		Average size of the particles (d), nm	***Saturation maximum (λ_{max}), nm***	***Sol color***
Hydrogen peroxide H_2O_2	1 : 1.6	6	514	Yellow-pink
Hydrogen peroxide H_2O_2	1 : 4	18	521	Pink-red
Sodium citrate $Na_3C_6H_5O_7$	1 : 4	16	520	Purple-red
Oxalic acid $C_2H_2O_4$	1 : 4	5	513	Orange-brown
Sugar $C_{12}H_{22}O_{11}$	1 : 500	80	553	Lilac-purple
Glycine $C_2H_5NO_2$	1 : 40	24	523	Raspberry-red

The rate of nucleation of a new phase depends on a degree of the solution oversaturation, i.e. mainly on the concentration of reacting substances and the chemical nature of the reducing agent. At a low nucleation rate and rather high particle condensation rate a small quantity of relatively

large-size particles is formed. At a higher nucleation rate and relatively low particle condensation rate the probability of the formation of an ample amount of the particles of a relatively small size is increased.

It should be noted that the sols obtained through the reduction of gold by hydrogen peroxide and stabilized with sodium citrate manifested the capacity for a long-term storage with the retention of their physical, chemical and biological properties [16]. In contrast to them, the sols obtained through the reduction of gold by oxalic acid turned out to be unstable due to the subsequent precipitation of the fine metallic gold powder on the bulb face. The capacity for the storage of the sols that were obtained through the reduction of gold by sodium citrate, sugar and glycerin can be considered as satisfactory taking into consideration the role of sodium citrate as a stabilizing agent.

From the standpoint of the minimum sizes of the nanoparticles and the stability of the solutions the gold gel obtained using hydrogen peroxide is of scientific interest in a greater degree. The reduction process of hydrogen tetrachloraurate (III) with hydrogen peroxide follows the reaction:

$$2HAuCl_4 + 3H_2O_2 = 2Au + 3O_2 + 8HCl.$$

The chemical interaction of the reagents results in appropriate color transformations: first, we observe the disappearance of the tint of yellow in the water solution of hydrogen tetrachoraurate (III) and the solution remains colorless for 1 or 2 seconds. Afterwards, the gold-containing suspension is formed that acquires the yellow-pink color. The opalescence is an inherent phenomenon of this colloid; the essence of it is that it remains transparent when visualized in the transmitted light and it seems to be turbid in lateral illumination (see Fig. 2). Such phenomenon of opalescence is explained by the scattering of the transmitted light by small colloid particles.

Colloidal particles can directly be observed using the electron microscope that gives us an opportunity to judge not only the number of particles and their motion but also their shape and structure. Fig. 3 gives the microimage of gold nanoparticles and it demonstrates the spherical shape and the sizes of the objects of the obtained nanosystem. The image clearly shows both large-size formations and monodispersed gold nanoparticles of ~ 6 nm. Citrate ions are adsorbed on the surface of gold nanoparticles and prevent their aggregation.

The antibacterial and antimycotic properties of the obtained gold sols were studied *in vitro* in the laboratories. The research data that are described in detail in [17] are indicative of that the preparations obtained during the experiment display an explicit antimicrobial activity both with respect to *Staphylococcus aureus* and *E.coli* and with respect to the fungi of a *Candida albicans* type (Fig. 4).

We also studied the antibacterial activity of gold nanoparticles with respect to the mixed microflora extracted from the oral cavity of dental patients that use partial dentures. The analysis of the sensitivity of the mixed microflora to gold sols showed that all the specimens of the nanoparticles have an explicit antimicrobial activity. The growth inhibition zones correlate with the data that were obtained during the studies of the reference strains of bacteria. Hence, it was established that actually all the colloidal gold solutions show a greater or lesser degree of sensitivity both to the reference test-strains of microorganisms and the mixed microflora of the patients with overdentures.

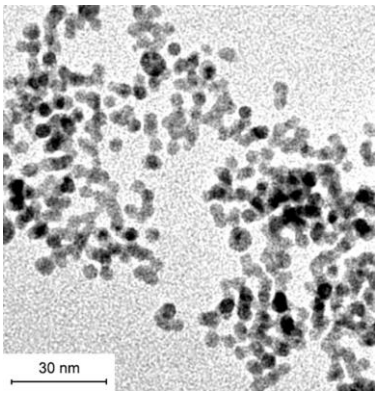

Fig. 3. The image of gold nanoparticles that were synthesized through the reduction by hydrogen peroxide and stabilized with the sodium citrate and this image was obtained using the transmission electron microscope.

Fig. 4. Petri dishes with the bacterial seeding (on the left) and the Sabouraud's medium (on the right) that were used for the determination of the sensitivity of the reference strains of microorganisms to the gold sol obtained through the reducing synthesis using hydrogen peroxide.

Based on the carried out microbiological investigations we estimated the efficiency of the colloidal gold solutions depending on the size of nanoparticles that were obtained through the reducing synthesis using different reagents. The appropriate diagram is given in Fig. 5. The diagram shows that the efficiency of the antimicrobial action of the preparations depends on the size (diameter) of gold nanoparticles, the smaller the particle size the stronger the manifestation of the properties of the particles and more effective the preparations prepared on their basis.

The investigation *in vivo* showed that the gold –based preparations with the particle size of ~ 6 nm turned out to be the most efficient for the treatment of mycotic lesions provoked by *Candida Albicans*. The preparations with particle sizes of 16, 18, 24 and 80 nm turned out to be of low efficiency.

The studies of the action of gold nanoparticles on the internal organs of experimental animals showed the following outcome: as for the kidneys, microscopically, the medullary and cortical substances are clearly differentiated,

interstitial edema is feebly marked, renal corpuscles are within histological norm, the epithelium is retained, the blood vessels are moderately full-blooded; as for the liver, the liver architectonics has been retained with specific lobular zoning, the venous sinuses are somewhat enlarged, the vessels are nonuniformly full-blooded, the bile ducts are moderately enlarged within the histological norm; and as for the heart we observe the feebly-marked interstitial edema, hypertrophy of single cardiomyocytes and the large vessels are within histological norm.

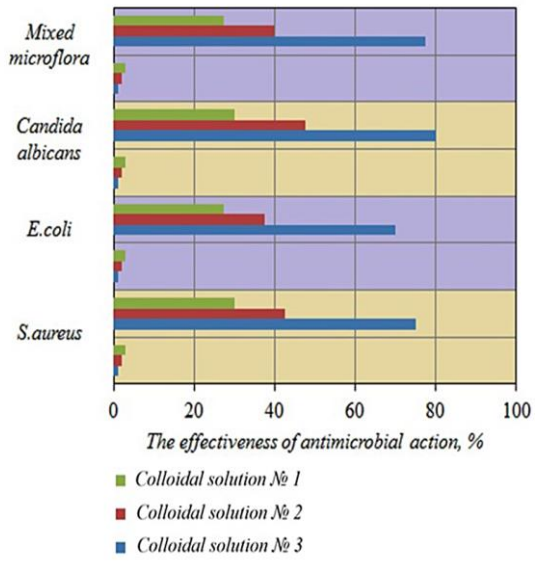

Fig. 5. The efficiency of antimicrobial and antimycotic action of the preparations based on colloidal gold solutions: 1 – sol obtained using glycine; 2 – sol obtained using the sodium citrate; 3 – sol obtained using the hydrogen peroxide.

The state of the internal organs of the studied rabbits showed similarity of the findings between group's № 2 and № 3. However, animals of group № 2 were found to have slightly thickened pericardium in a state of mild edema and small-focal, mildly expressed infiltration by cells of the lymphoplasmacytic series with single mononuclear cells. A number of hepatocytes in animals of group № 3 were shown to have small-focal clarifications of the cytoplasm with clumps of glycogen. The investigation *in vivo* showed the unavailability of an essential negative effect of gold nanoparticles on internal organs of animals and it gives us premises for the introduction of the obtained medical preparations to the treatment protocols of fungous lesions.

VI. Conclusions

Thus, the use of reducing synthesis as a method for obtaining gold nanoparticles is characterized by simplicity of the technological process, purity and reliability of the prepared therapeutic agents. For chemical synthesis, the authors used only non-toxic reagents. The findings of the study offer new data on the effect of the nature of reducing agents (Hydrogen peroxide, Sodium citrate, Oxalic acid, Sugar, Glycine) and the ratio of reagents during synthesis on the size of gold nanoparticles and on the degree of microbiological activity of therapeutic agents based on them. It has been established that the biological effect of gold nanoparticles is significantly enhanced with a decrease in their size from 80 to 6 nm. The resulting therapeutic agents have passed *in vitro* and *in vivo* laboratory studies. The results of the study are positive and coincide with the conclusions made by other authors in [17, 18]. In general, the research demonstrates a high level and a wide range of antimicrobial and antimycotic effects of gold nanoparticles, absence of their toxic effects on the function of vital organs, and good biocompatibility.

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