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Variability of pathogenicity factors representative of the human microbiome under the influence of γ-Fe_2O_3 nanoparticles

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Abstract

Biomedical applications of nanoparticles require deep understanding of their interaction with normal human microflora. Previously, the toxic and mutagenic properties of iron oxide nanoparticles as well as their effect on the growth and morphology of the microflora were extensively investigated. However, the studies related to the variability of microbial pathogenicity factors induced by iron oxide nanoparticles are very limited. Meanwhile, this characteristic of microbes is genetically determined and is important for their survival and distribution in the human body. Therefore, pathogenicity factors are significant indicators of the experimental studies. In this work, the effect of the presence of Fe_2O_3 nanoparticles obtained by laser target evaporation (LTE) on selected enzymes that demonstrate invasion and aggression factors was evaluated for three reference strains of *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*. It was found that the presence of LTE Fe_2O_3 nanoparticles supplied in the form of water-based suspensions does not induce changes of the above-mentioned parameters.

Keywords

laser target evaporation
iron oxide nanoparticles
magnetic nanoparticles
biomedical applications
eukaryotic and prokaryotic microorganisms
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1. Introduction

Magnetic nanomaterials are moving from the stage of scientific research to the stage of practical implementation in diagnostics, therapy and biotechnologies [1, 2]. Many studies have been associated with proposals to use nanomaterials for targeted drug delivery, creating bone implants, wearable devices, etc. Magnetic nanoparticles (MNPs) are especially attractive as their movements inside the body can be controlled by application of gradient of the external magnetic field [3, 4], which can be also used to induce hyperthermia and thermal ablation [5].

Magnetic nanoparticles can be produced by different chemical and physical techniques. Interestingly, about 92% of MNPs of iron oxides are prepared by chemical routes: co-precipitation, hydrothermal and solvothermal synthesis, sol-gel and polyol microemulsion methods, microwave-assisted synthesis electrochemical deposition and others [2, 6, 7]. About 6% of MNPs of MNPs are fabricated by physical techniques [8, 9], and about 2% by biological synthesis methods [2, 10]. The largest batches of the MNPs can be obtained using electrophysical techniques such as electric explosion of the wire, laser target evaporation or spark discharge [2, 9]. In any case, biomedical applications require synthesis of large batches, since established protocols demand testing with an extended set experimental technique [11]. In addition, biological and biomedical applications are only possible with aqueous suspensions (not with the air-dry MNPs). Their fabrication also to some extent reduces the amount of available material.

On the one hand, the composition of nanoparticles includes natural components: iron, silver, copper, aluminum, oxygen, etc. On the other hand, their size, shape, exact composition, and physicochemical properties make it possible to classify nanoparticles as xenobiotics, i.e. chemical substances found within an organism that is not

naturally produced or expected to be present in it. The question about the degree of safety of each particular nanomaterial for organisms and ecosystems in general is a very important one to be answered.

An analysis of the experimental results presented in the scientific sources allows to distinguish three groups of effects of nanoparticles on biological systems: modifications, toxicity, and mutagenicity. For example, a change in the color of eukaryotic organisms was established in an experiment with iron-containing nanoparticles [12]. Iron nanoparticles caused reversible changes in the biochemical activity of *Pseudomonas aeruginosa*: a decrease in carbohydrate fermentation was observed [13].

The toxic effects of nanoparticles were found for organisms of different systematic groups [14]. Copper nanoparticles caused the death of bacteria. Nanoparticles based on aluminum oxide reduced the ability of *Escherichia coli* (*E.coli*) to form a biofilm [15]. Iron nanoparticles had a toxic effect on *Pseudomonas aeruginosa* [13]. The low toxicity of nanoparticles of silicon dioxide was shown for some strains of *E.coli* [16]. Toxic effects were also found in different types of eukaryotic organisms [14, 17, 18].

Deoxyribonucleic acid (DNA) damage, including mitochondrial DNA, and the formation of micronuclei in blood cells can be induced by silicon, nickel, and gold containing nanoparticles. Metal nanoparticles cause excessive production of reactive oxygen species (peroxides, superoxide, singlet oxygen, etc.), which induce DNA damage [14]. The mutagenicity of aluminum oxide nanoparticles for human cells was also proven [19]. It is important to take into account that, regardless the type of nanoparticles and biotests used in the experiment, the first interaction of the agent occurs with the normal microflora of the body, regardless of the method of administration.

Previously, the toxic and mutagenic properties of iron oxide nanoparticles as well as their effect on the growth and morphology of the microflora were extensively investigated. However, up to now, the studies of the microbial variability of pathogenicity factors related to the presence of the iron oxide nanoparticles were quite limited. The variability of pathogenicity factors is both genetically determined and important for the microbial survival and distribution in the body. Therefore, pathogenicity factors are significant indicators of the experimental studies.

In this work, the effect of the presence of iron oxide nanoparticles obtained by laser target evaporation supplied as water-based suspension was studied for selected enzymes that demonstrate invasion and aggression factors. Three reference strains of *Candida albicans, Staphylococcus aureus,* and *Escherichia coli* were used.

2. Materials and methods

Electrophysical techniques allow fabrication of large batches of MNPs (of the order of 100 g) required for biomedical research and applications [9]. One of these techniques is the laser target evaporation (LTE) in which the solid target is evaporated by the high-power pulse of a laser beam. The beam energy transform into the kinetic energy of the evaporated products. In this study, the LTE target was fabricated using the commercial Fe_3O_4 iron oxide powder (Alfa Aesar). More details of the experimental procedure for the LTE synthesis can be found elsewhere [9].

The X-ray diffraction investigation was performed using s DISCOVER D8 Bruker diffractometer and TOPAS software allowing Rietveld full-profile refinement for the quantitative analysis. Transmission electron microscopy (TEM) was performed for the evaluation of the size and shape of MNPs (JEOLJEM2100). For the measurements of the specific surface area (S_{sp}) of the MNPs ensemble, the low temperature sorption of nitrogen technique was used [1]. Magnetic properties were studied with a MPMSXL-7 SQUID magnetometer.

Biomedical applications require supplying magnetic MNPs as water-based suspensions. Electrostatically stabilized water-based suspension of MNPs was prepared with sodium citrate in 5 mM concentration via disaggregation by ultrasound treatment on a Cole-Parmer CPX-750 homogenizer at the power output 300 W and centrifuging using a Hermle Z383 apparatus in order to remove the largest aggregates. The final concentration of MNPs in the stock suspension was 47.8 g/l, and the suspension was slightly acidic (pH=4.5) due to the specific adsorption of hydroxide ions on the surface. The stock suspension was then diluted with distilled water to provide the desired concentration of MNPs therein. Hydrodynamic diameters of the MNPs and their aggregates were defined by dynamic light scattering technique (Brookhaven Zeta Plus). The same instrument allowed measuring the electrokinetic zeta-potential of the suspensions by the electrophoretic light scattering.

The fungus strain *Candida albicans* ATCC 10231 [20], *Staphylococcus aureus* ATCC 25923 [21] and *Escherichia coli* ATCC 25922 [22] strains, which belong to the normal human microflora, were used as bioassays. In the control group, the bacteria grew in a liquid nutrient medium MPA (meat-peptone agar), the pH of which was 7.4±0.4. The fungi were cultivated in a liquid Sabouraud medium (pH 5.7±0.2). In the experimental group, a suspension of MNPs was introduced into the liquid medium, the concentration of which reached 0.1, 1.0 and 10.0 MPD (maximum permissive dose) for Fe^{+3}. The microbial suspension was exposed for 144 hours for all groups.

The experiments included the following stages. Starting from the zero point, every 24 hours, microorganisms were inoculated from the culture liquid onto a solid nutrient medium (MPA, and Nickerson agar). Several clones were selected by random sampling among the grown colonies to determine pathogenicity factors. The clones were placed onto differential diagnostic media and cultivated at a temperature of 37 ° C for 24 hours. After that, the presence or absence of pathogenicity factors was recorded

similar to the way reported in the literature [23–25]. The analyzed indicators (pathogenicity factors) were provided by the presence of certain enzymes in microbes and their activity. Respiratory, lecithinase, plasmacoagulase, hemolytic, and DNAase activities were taken into account.

Respiratory activity is assessed by changing the color of the culture in the medium with the dye Congo red. Lecithinase activity is assessed by the formation of a cloudy "corolla" around the colony on a nutrient yolk medium. The plasmacoagulating activity of *S. aureus* is determined by the presence of a gelatinous formation. Hemolytic activity is manifested through the formation of a zone of enlightenment on a nutrient medium. Determination of DNAase activity is associated with the formation of a cleavage zone, i.e. zones of enlightenment of the nutrient medium around the colony.

It should be noted that qualitative indicators were collected; therefore, their presence "+" or absence "–" was recorded. Depending on the severity of the symptom, the following states were noted: "–" – the indicator is negative, "+" – low activity, "++" – moderate activity, "+++" – pronounced activity, "++++" – maximum activity.

3. Results

X-ray diffraction analysis showed that obtained iron oxide powder have the inverse spinel structure (Fd-3m space group). According to the TEM studies, which included the graphical analysis (Figure 1), the shapes of the MNPs were very close to being spherical. The nanoparticles in the ensemble were size-distributed in accordance with the lognormal law with 17±3 nm average mean diameter. According to PSD, 99% of MNPs fell within the diameter range of 2–40 nm. The specific surface area was about 69 m^2/g. Magnetic measurements confirmed that nanoparticles had low coactivity of about 30 Oe at room temperature and the saturation magnetization of about 37±2 emu/g (both numbers are consistent with existing sources for the Fe_2O_3 MNPs of this size) [1, 9].

The average hydrodynamic diameter, 51.4±0.4 nm, was determined for the species in MNPs suspension. It was higher than that for the air-dry MNPs. It meant that the suspension was in fact a mixture of individual MNPs and their small aggregates. The value of zeta-potential of the suspension was –44±2 mV, which was well above the coagulation threshold (20 mV regardless of the sign). It meant that the aggregates were stable and no further aggregation occurred in the suspension. These parameters of the suspension did not change in storage for over a year.

Figure 2 shows the general view of the samples of the control groups of the fungus strain *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 strains, belonging to the normal human microflora.

In order to determine the effect of MNPs on the variability of pathogenicity factors, 144 clones were isolated from the eukaryotic strain *C.albicans* ATCC 10231, 144 clones from the gram-positive prokaryotic strain *S.aureus* ATCC 25923 and 83 clones from the gram-negative prokaryotic strain *E.coli* ATCC 25922.

It was found that the reference strains under physiological conditions (control) retain a set of specific aggression factors (Table 1), which they implement in a low virulent form.

This corresponds to the levels of activity that are capable of being manifested by the microorganisms of these species, which are part of the structure of the normal human microbiome, without leading to the development of pathological processes in internal organs and tissues. This result is evidenced by the absence of DNase and hemolytic activities.

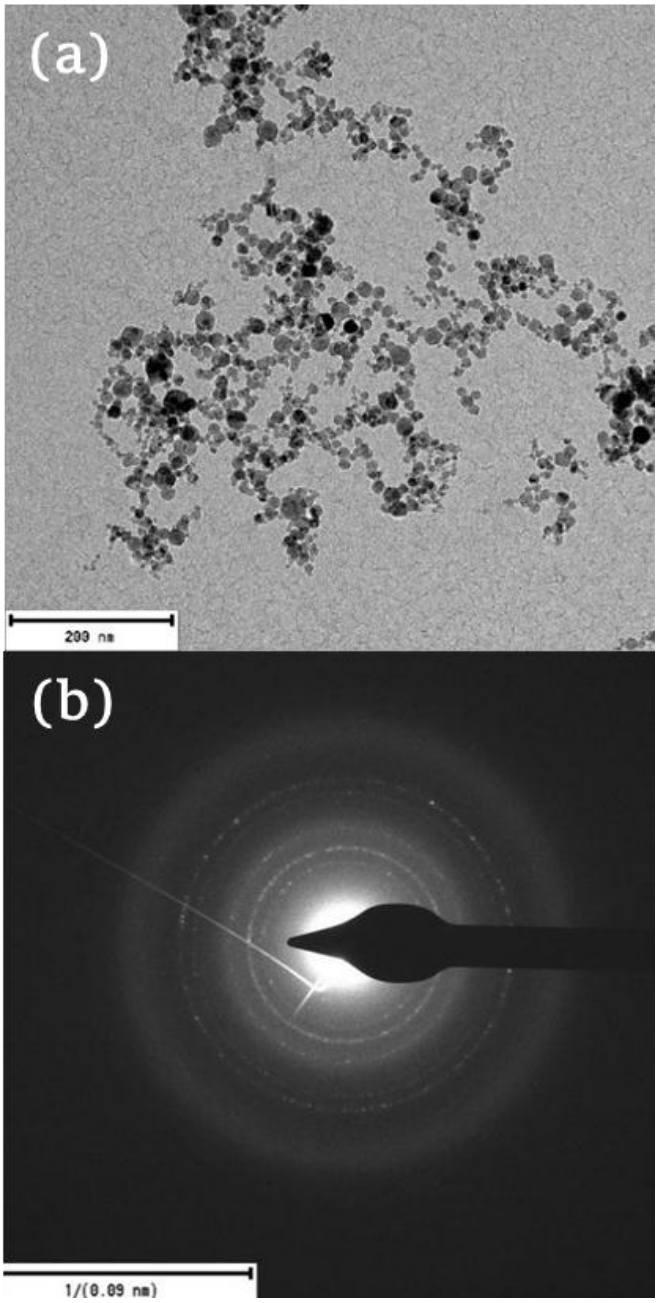

Figure 1 Transmission electron microscopy: general view of γ-Fe_2O_3 LTE nanoparticles (a), TEM microdiffraction confirming spinel structure (b).

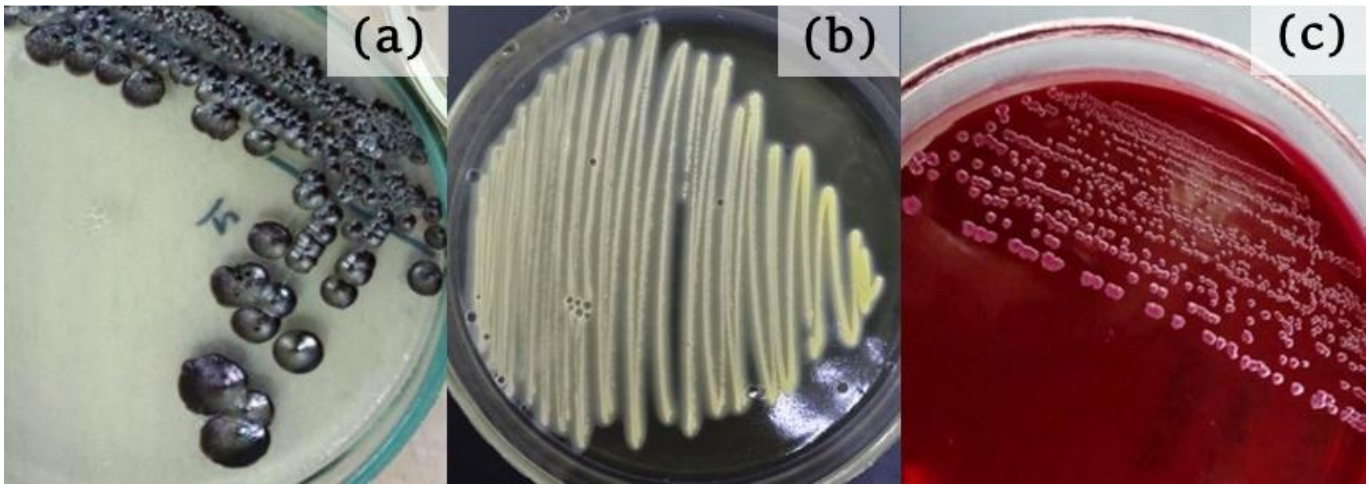

Figure 2 *Candida albicans* on Nickerson's nutrient medium in Petri dish (a), *Staphylococcus aureus* on nutrient medium yolk-salt agar (b), *Escherichia coli* on ENDO nutrient medium (c).

Table 1 Evaluation of pathogenicity factors in reference strains in the control group.

Enzymatic activity	Test objects		
	C. albicans	*St. aureus*	*E. coli.*
Respiratory	++	++++	++++
Lecithinase	–	+	–
Plasmocoagulase	–	+++	–
Hemolytic	–	–	–
DNAase	–	–	–

The presence of lecithinase and plasma-coagulase activity, due to which they are able to realize their immunogenic properties in the human body, indicates the normally functioning work of plastic metabolism. Respiratory activity in microorganisms is an indicator of a normally functioning energy metabolism system. The results of the experiments on the variability of factors of invasion and aggression of microorganisms in the presence of nanoparticles are shown in Table 2.

It was found that the activity of the reference strains of microorganisms after exposure to the nanoparticles does not change in all variants of the experiment. DNase and hemolytic activities were not detected. Respiratory activity also remained unchanged in all variants of the experiment (Figure 3a).

Lecithinase and plasmacoagulase activities were found only in staphylococcus, being at the same level as in the control group. C. albicans shows only respiratory activity (Figure 3b). Thus, the factors associated with the level of virulence of microorganisms remain stable in the experiment and do not differ from the control level. Perhaps this is due to the fact that nanoparticles cannot affect the metabolic processes of a microbial cell due to their adhesion to the cell wall.

4. Conclusions

Iron oxide Fe_2O_3 nanoparticles were obtained by the LTE technique. They had the inverse spinel structure (Fd-3m space group), close to spherical shapes and the lognormal size-distribution, and they had 17±3 nm average mean diameter and magnetic parameters consistent with their size and composition. Biological experiments resulted in the following findings:

1. DNase and hemolytic activities were not detected in all test samples, neither in the control nor in the experimental groups.
2. Lecithinase and plasmacoagulase activities were recorded only in *S. aureus* in the control and in all experimental groups at the same level.
3. *C. albicans* showed moderate respiratory activity, and *S. aureus* and *E. coli* showed pronounced respiratory activity. The control and all experimental groups did not differ from each other.

Table 2 Evaluation of pathogenicity factors in reference strains obtained in the experiments.

Test objects	MTD calculated on the basis of Fe^{+3} content in MNPs	Enzymatic activity				
		DNAase	Respiratory	Hemolytic	Lecithinase	Plasmocoagulase
C. albicans	0.1	–	++	–	–	–
	1.0	–	++	–	–	–
	10.0	–	++	–	–	–
St. aureus	0.1	–	++++	–	+	+++
	1.0	–	++++	–	+	+++
	10.0	–	++++	–	+	+++
E. coli.	0.1	–	++++	–	–	–
	1.0	–	++++	–	–	–
	10.0	–	++++	–	–	–

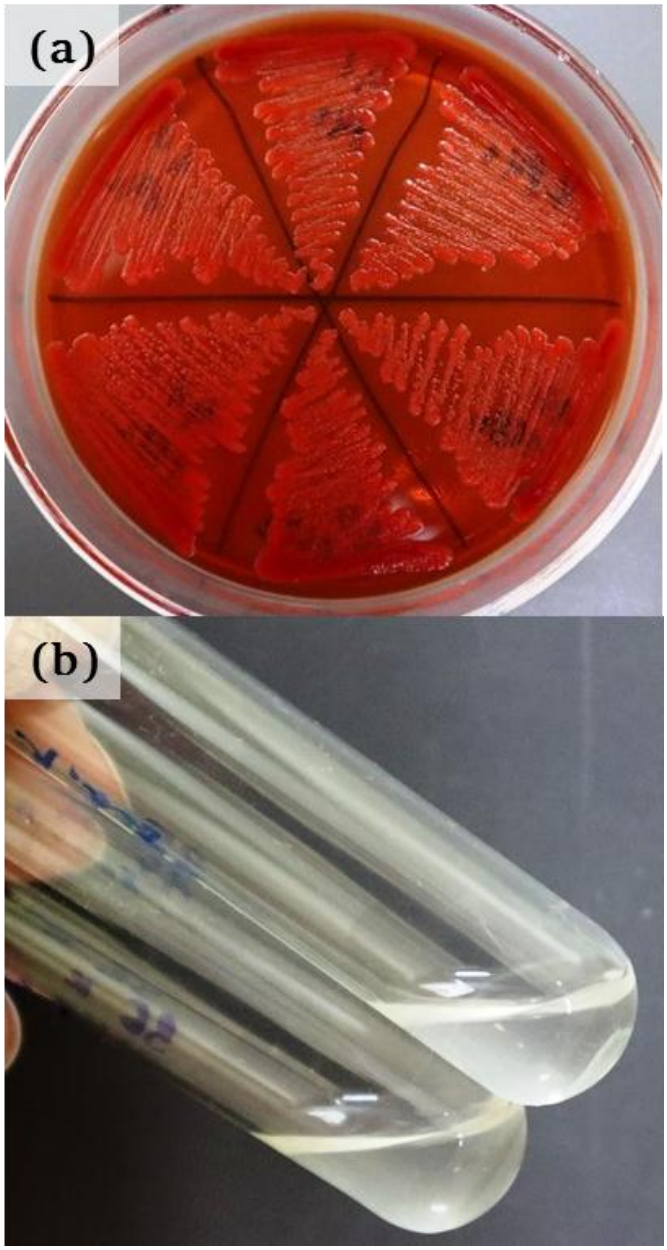

Figure 3 Pronounced respiratory activity of staphylococcus (a), a negative test for plasmocoagulase in *C. albicans* (no clot is formed) (b).

4. The induced variability of indicators of pathogenicity factors in the experiment was not established. Given that pathogenicity factors are the genetically determined traits, it can be assumed that the studied nanoparticles either do not penetrate into the bacterial cell or have no effect on the genetic material.

Supplementary materials

No supplementary materials are available.

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Conflict of interest

The authors declare no conflict of interest.

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