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A Bacterial Enzymatic System Neutralizes the Impact of Silica-Magnetite Nanocomposites on ROS Levels

This study investigates the reactive oxygen species (ROS) generation and biological activity of novel silica-magnetite nanocomposites, TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4, where TA is a silicon dioxide copolymer, AA is ascorbic acid, and HA is humic acids. A key challenge in nanotoxicology is the contradictory data from complex biological systems. To address this, we employed a standardized bioluminescent enzymatic assay (bacterial luciferase/oxidoreductase) as a simple, rapid biosensor system to evaluate the nanocomposites' effects under controlled conditions. We compared ROS activity in both non-biological (enzyme-free aqueous solutions) and biological (enzymatic system) environments, with and without model oxidative stress induced by 1,4-benzoquinone. The enzymatic system exerted a pronounced neutralizing effect on ROS content, suppressing the significant ROS fluctuations and synergistic ROS generation (up to 300%) observed in non-biological media in the presence of the oxidizer. Additionally, the nanocomposites showed no effect on the bioluminescence intensity of the enzymatic system. This disparity between the higher reactivity in simple aqueous solutions and the neutral effect in the enzymatic system highlights the critical role of biological matrixes. The findings suggest that enzymatic environments can mitigate the radical processes driven by iron-based nanocomposites, which is crucial for predicting their biological activity and potential for applications like ferroptosis-based tumor therapy.

Keywords: iron-based nanocomposite, magnetite, silica, ascorbic acid, humic acids, bacterial enzymatic assay, reactive oxygen species, bioluminescence, chemiluminescence

Introduction

The biological activity of nanoparticles is a highly promising field of study, and the number of articles and reviews in this field is growing rapidly [1–3]. However, the results of these studies are often contradictory due to the variety of experimental conditions and the use of complex biological systems. This makes it difficult to compare and predict the properties of nanoparticles.

Superparamagnetic iron oxide nanoparticles (Fe_3O_4) are widely used for adsorption [4], enzyme immobilization [5], Fenton reaction catalysis [6–8], drug delivery [9], and energy storage [10–11]. To prevent the aggregation of these nanoparticles [12], their surfaces are modified with various organic and inorganic ligands, including humic acids (HA), ascorbic acid (AA) and polymer derivatives of silicon dioxide—tetraethoxysilane and 3-aminopropyltriethoxysilane (TA) [13–15].

Nanocomposites based on silica-magnetite nanoparticles (TA-Fe_3O_4) are promising for biomedical applications due to their biocompatibility, magnetic properties and easy surface functionalization [16-17]. Mesoporous silica nanoparticles enable flexible tuning of their structure for efficient drug loading and targeted delivery. They also serve as substrates for other nanomaterials, forming multifunctional composites.

AA and HA act as surface modifiers, preventing aggregation and influencing Fenton reaction-driven ROS generation by iron-containing nanoparticles [18–21], resulting in iron-programmed cell death—ferroptosis. AA maintains magnetite stoichiometry and has both antioxidant and prooxidant effects, while

HA enhance the catalytic activity of Fe_3O_4 in Fenton reactions. On the other hand, magnetite can provide magnetically-controlled delivery of AA and HA in organisms.

Previous research showed that nanocomposite Fe_3O_4-AA-MOF (where MOF is a metal-organic framework) retained the antibacterial and prooxidant properties of AA [22]. In this system, MOF-Fe_3O_4 acts as an inert magnetic carrier.

Despite the fact that some silica-based nanostructures have passed clinical trials, concerns about their safety persist. The key challenges include conducting toxicity studies, designing simple but effective nanoplatforms, and understanding their *in vivo* mechanisms. The toxicity of mesoporous silica nanoparticles depends on their size, morphology [23-24], surface charge [25], degree of crystallinity [26–30], and concentration [31]. Studies report that membrane damage, cytotoxicity, hemolysis [32-33], and immune activation [34] depend on nanoparticle properties and cell type.

However, the available data pertain to various aspects of the biological activity of nanomaterials and are typically obtained under non-comparable conditions. The standardization of experimental protocols remains an urgent task. For this purpose, the authors selected a simple and rapid biosensor system—a bioluminescent enzymatic assay.

The bacterial luminescence bioassay is widely used in ecological studies. This technique exploits the fact that luminous marine bacteria naturally produce light; thus, a decrease in luminescence intensity directly indicates the presence of toxic substances. High sensitivity, speed, and simplicity have made this bioassay a standard tool for toxicity monitoring for more than six decades [35–38]. The long-term application of this bioassay has contributed to a broad understanding of the effects of various compounds on bacterial cells, their enzymes, and cellular membranes [39–41]. Patterns of the effects of toxicants on enzymatic reactions contribute to understanding the mechanisms of toxic effects on living organisms.

A newer approach involves using the isolated enzymes responsible for the luminescence. In 1990, a toxicity bioassay was developed based on a coupled enzyme system including bacterial luciferase and NAD(P)H:FMN-oxidoreductase [42]:

$$NADH + FMN \xrightarrow{\text{NAD(P)H:FMN-oxidoreductase}} NAD^+ + FMNH^- \quad \text{(R.1)}$$

$$FMNH^- + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOO^- + H_2O + h\nu\ (490\ \text{nm}) \quad \text{(R.2)}$$

This enzyme system is sensitive to redox-active compounds; its bioluminescence depends on concentration and redox parameters of these compounds [39-40, 43]. Both enzymatic and bacterial bioluminescence assays have been used to study the prooxidant [20, 44] and antioxidant [45–49] properties of bioactive compounds, and to detect the involvement of reactive oxygen species (ROS) in these effects.

ROS are oxygen-containing molecules with unpaired electrons, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), etc. They are constantly present at physiological levels and play key roles in cellular signaling [50] and apoptosis [51–54]. While essential for normal metabolism, excessive ROS levels produce toxic effects, damaging DNA, proteins, and lipids [55]. Maintaining ROS balance is, therefore, vital for cellular function.

In bioluminescence systems, ROS are both generated and utilized in the coupled redox reactions. Therefore, the role of ROS in these enzymatic systems (Reactions R.1 and R.2) is crucial.

Iron's ability to generate ROS is directly due to its unique chemical properties: partially filled *d*-orbitals, variable oxidation states, and involvement in electron transfer processes. Investigating the ROS-related activity of iron nanoparticles, such as magnetite-silica nanocomposites, is crucial for developing their biomedical applications.

Our study aims to characterize silica-based nanocomposites (TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4). We investigated ROS generation and the biological activity of these compounds, comparing their ROS activity in a non-biological system and the simplest biological system (an enzymatic reaction). The results will contribute to predicting the biological activity of iron nanoparticles, which is important for the development of ferroptosis-based tumor treatments.

Experimental

Preparation of a Copolymer of Tetraethoxysilane and 3-Aminopropyltriethoxysilane (TA)

To prepare the copolymer, 150 mL of deionized water (DI) was added to a mixture of 10 mL of tetraethoxysilane (T) and 4.2 mL of 3-aminopropyltriethoxysilane (A) (molar ratio T:A = 1:0.5) under constant stirring on an overhead mixer (600 rpm, 10 min). The mixture was then stirred on an orbital shaker

(150 rpm, 24 h) at room temperature. The resulting precipitate was collected and washed with DI water until the supernatant reached pH~8. The final product was dried and lyophilized in a freeze dryer at –37 °C.

Preparation of TA-HA-Fe_3O_4 Composite

To obtain the TA-HA-Fe_3O_4 composite (1:0.1:1 wt.%), 2 g of TA and 0.2 g of HA were dispersed in 200 mL of DI in an argon atmosphere on an overhead agitator (1400–1500 rpm, 30 min). The concentrations of magnetite nanoparticles, as a source of iron ions, were determined by calculating a 20-fold excess of Fe^{3+} (HA-Fe = 1:20) based on the content of HA functional groups (5 mmol/g of the COOH and OH groups), in order to obtain water-soluble humic preparations with a high iron content. As a result, salt mixtures of 5.4 g of $FeCl_3 \cdot 6H_2O$, 1.98 g of $FeCl_2 \cdot 4H_2O$ and 12 mL of 25 % NH_4OH were added. The formed precipitate was separated on a paper filter, washed with DI and dried in a dynamic vacuum. Wt.% Fe: 37.7.

Preparation of TA-AA-Fe_3O_4 Composite

To obtain the TA-AA-Fe_3O_4 composite (1:0.1:1 wt.%), iron salt samples were dissolved in DI in an argon atmosphere and 12 mL of 25 % NH_4OH was added with constant stirring (1400–1500 rpm, 30 min). The formed Fe_3O_4 precipitate was separated using a magnet (0.3 T) and washed once with 200 mL of DI. Then 0.2 g of AA was dissolved in 70 mL of suspension containing Fe_3O_4, 200 mL of 96 % C_2H_5OH (pH = 7–8) was added with constant stirring (1200 rpm) and 2 g of TA. The solution was stirred on an overhead mixer (1000 rpm) for 60 minutes. The formed precipitate was separated on a paper filter, washed with DI and dried in a dynamic vacuum. Wt.% Fe: 51.8.

Characterization of Nanocomposites

Samples of the nanocomposites TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4 were characterized using X-ray diffraction, transmission electron microscopy, and low-temperature nitrogen adsorption (Supplementary Materials A, B).

Luminol Chemiluminescence Assay System

ROS levels were quantified using a luminol-based chemiluminescence method. Measurements were performed both in distilled water (enzyme-free solutions) and within the enzymatic system, across a range of composite concentrations [56]. The chemiluminescence reaction was initiated by adding an alkaline luminol solution (3×10^{-5} M). The concentration of $K_3[Fe(CN)_6]$ in all samples was maintained at 1.2×10^{-4} M.

Bioluminescence Enzymatic Assay System

The effects of the silica-based composites on an enzyme system were evaluated using a bioluminescence enzymatic assay. The composition of this bioluminescence enzyme system is described in [56].

Measurements of Bioluminescence/Chemiluminescence Intensities

The preparation of nanocomposite suspensions for bioluminescent and chemiluminescent analyses was the same as described in [56].

a) Luminol Chemiluminescence Assay

To assess a role of ROS in the biological effects of the nanocomposites, the chemiluminescence intensity was measured in the enzyme-free and enzymatic solutions.
The relative ROS content (ROS^{rel}) was defined as follows:

$$ROS^{rel} = ROS_{NC}/ROS_{contr}, \quad (1)$$

where ROS_{contr} and ROS_{NC} are the ROS contents in the absence and presence of nanocomposites, respectively.

Additionally, ROS contents were determined under conditions of model oxidative stress. To model oxidative stress, we employed the organic oxidizer 1,4-benzoquinone (Sigma-Aldrich, St. Louis, MO, USA). For the experiments, a benzoquinone concentration that inhibits the bioluminescence intensity by 50 % ($IC_{50} = 10^{-6}$ M) was selected. The relative ROS content (ROS_{Bq}^{rel}) was defined as follows:

$$ROS_{Bq}^{rel} = ROS_{NC+Bq}/ROS_{Bq},$$

where ROS_{NC+Bq} and ROS_{Bq} are the ROS contents in 1,4-benzoquinone solutions in the presence and absence of nanocomposites, respectively.

b) Bioluminescence Enzyme System

The inhibitory effects of the nanocomposites on the enzymatic activity were quantified using the relative bioluminescence intensity, I^{rel}:

$$I^{rel} = I_{NC}/I_{contr}, \quad (3)$$

where I_{contr} and I_{NC} are the maximum bioluminescence intensities in the absence and presence of nanocomposites, respectively.

The effects of the nanocomposites in the enzymatic bioluminescent system under model oxidative stress were evaluated using relative bioluminescence intensity, I_{Bq}^{rel}:

$$I_{Bq}^{rel} = I_{NC+Bq}/I_{Bq}, \quad (4)$$

where I_{NC+Bq} and I_{Bq} are the maximum bioluminescence intensities in 1,4-benzoquinone solutions in the presence and absence of nanocomposites, respectively.

Statistical Processing

Bio/chemi-luminescence measurements were performed in 5–10 replicates at 25 °C using a Luminoskan Ascent bioluminometer (Thermo Electron Corporation, USA) with an integrated injector. The maximum chemiluminescence and bioluminescence intensities were determined. Standard deviations (SD) for I^{rel} and ROS^{rel}, I_{Bq}^{rel} and ROS_{Bq}^{rel} were analyzed with GraphPad Prism 8 (GraphPad Software, Inc., USA); all SD were less than 20 %. To evaluate quantitatively the spread of data across experimental curves, we compared the coefficients of determination (R^2) for the exponential dependences of I^{rel}, ROS^{rel}, I_{Bq}^{rel} and ROS_{Bq}^{rel} on nanocomposite concentrations.

Results and Discussion

A. ROS Content in Water Suspensions of TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4. Enzyme-Free and Enzyme Solutions

Preliminarily, we studied the effects of the triple nanocomposites TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4 on ROS content in distilled water (Fig. 1A). The figure shows no significant effects ($p > 0.05$) at nanocomposite concentrations $<10^{-2}$ mg/L and a minor suppression of ROS content at concentrations $>10^{-2}$ mg/L.

We simulated a biological medium in the nanocomposite suspensions by adding the enzyme system. The ROS content was measured during the enzymatic bioluminescence process. Figure 1C shows that the addition of the enzymes did not notably change the ROS content at low nanocomposite concentrations ($< 10^{-2}$ mg/L) and mitigated the minor ROS suppression observed at higher concentrations ($> 10^{-2}$ mg/L).

However, the data scatter across the curves reduced compared to the enzyme-free medium (Fig. 1*A*). The values of the coefficients of determination (R^2) for the exponential dependences of ROS^{rel} on nanocomposite concentration increased from 0.02 to 0.14 for TA-AA-Fe_3O_4 and from 0.02 to 0.34 for TA-HA-Fe_3O_4. This increase quantitatively confirms the reduced variation in ROS content when replacing the enzyme-free medium with the enzymatic one.

Additionally, we conducted similar experiments in the presence of a model oxidizer—1,4-benzoquinone. In our previous research [20, 22, 44, 56], we used this compound to model oxidative stress in bacterial suspensions and enzymatic solutions. This approach is based on the assumption that such model oxidative stress stimulates the additional formation of active oxygen radicals (in accordance with the Fenton reaction [6–8]) and, consequently, may induce antitumor activity.

Figure 1*B* shows the ROS content in aqueous solutions at different concentrations of TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4 nanocomposites in the presence of 1,4-benzoquinone. The difference from the medium containing no benzoquinone (Fig. 1*A*) is pronounced: the presence of 1,4-benzoquinone increased the ROS content by up to 150 % for TA-HA-Fe_3O_4 and by up to 300 % for TA-AA-Fe_3O_4 across the entire concentration range studied (Fig. 1*B*). The 300 % increase in ROS content in TA-AA-Fe_3O_4 suspensions (Fig. *1B)* can be explained by synergistic effects on ROS generation via a process known as redox cycling [57]. According to the authors' findings, quinones, ascorbic acid, and iron interact in redox cycles involving semiquinone radical intermediates, iron participation, and OH generation through Fenton reactions. The more moderate increase in ROS content observed in TA-HA-Fe_3O_4 suspensions (Fig. 1*B*) may be related to processes discussed in [58-59]: humic acids (HA) and quinones can accelerate Fe(III) reduction by electron shuttling, and the subsequent ROS generation via the Fenton reaction may result from this acceleration.

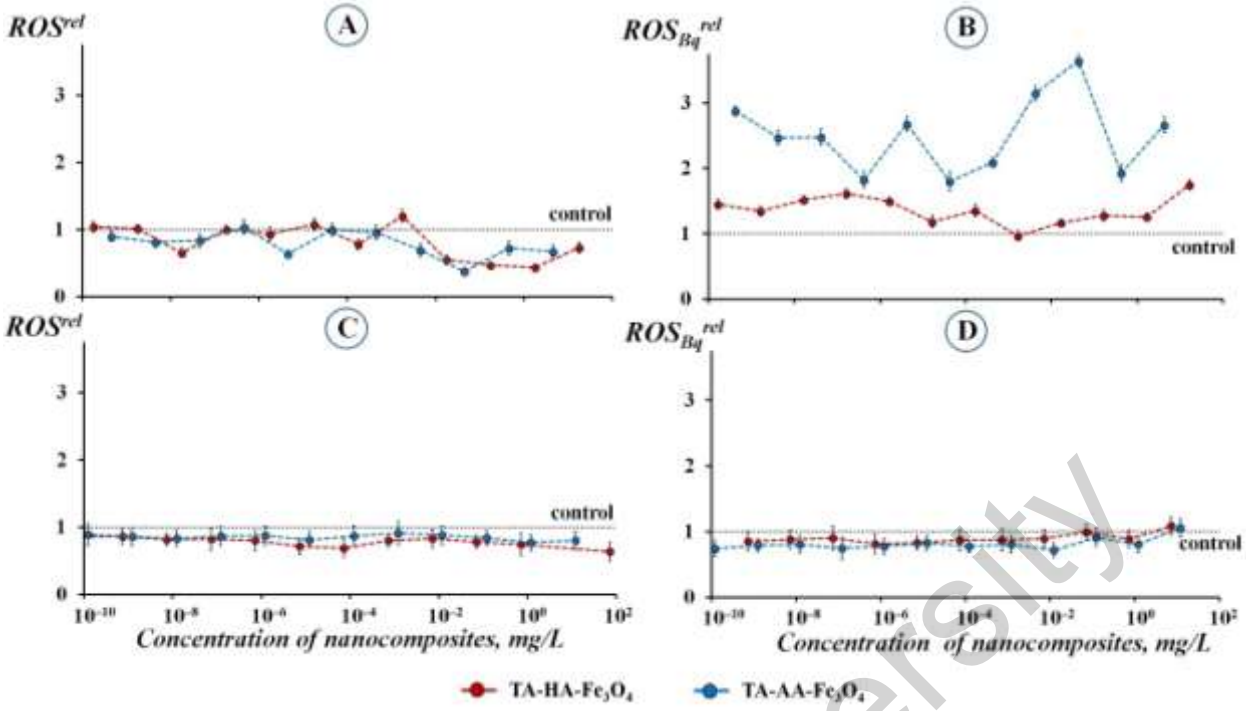

Figure 1. Relative ROS content: (*A*) in enzyme-free water solutions, ROS^{rel};
(*B*) under conditions of oxidative stress in enzyme-free water solutions, $ROS_{Bq}{}^{rel}$;
(*C*) in the enzyme system, ROS^{rel}; (*D*) under conditions of oxidative stress in the enzyme system, $ROS_{Bq}{}^{rel}$.
Control suspension — in the absence of nanocomposites.
Conditions of oxidative stress — in the presence of 1,4-benzoquinone ($IC_{50Bq} = 10^{-6}$ M).

Figure 1*D* presents the ROS content in the enzymatic system in the presence of 1,4-benzoquinone (10^{-6} M) at different concentrations of the nanocomposites. A comparison of Figures 1*B* and 1*D* reveals a neutralizing effect of the enzymes on the ROS content: the addition of nanocomposites TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4 did not alter the ROS content compared to the control solution under conditions of model oxidative stress.

Additionally, the data spread across the curves in Figure 1*D* decreased compared to the enzyme-free medium (Fig. 1*B*), similar to the trend observed in benzoquinone-free media (Figures 1*A* and 1*C*). The values of the coefficients of determination (R^2) for the exponential dependencies of ROS^{rel} on nanocomposite concentration increased from 0.01 to 0.40 for TA-AA-Fe_3O_4 and from 0.10 to 0.38 for TA-HA-Fe_3O_4. This increase again quantitatively confirms the reduced variation in ROS content when replacing the enzyme-free medium with the enzymatic one.

The fluctuations in ROS levels observed in Figures 1*A* and 1*B* show a non-random pattern, reminiscent of effects discussed in the literature [60]. In any case, the effect noted in our study (Fig. 1*B*) is interesting and warrants further investigation. Importantly, we have observed similar ROS fluctuations for other types of magnetite-based nanocomposites, specifically Fe_3O_4-AA-MOF and Fe_3O_4-HA-MOF, in an aquatic non-biological environment (Supplementary Materials C, Figures S5A, S5B).

Hence, we observed a neutralizing effect of the enzymatic system on the ROS content in suspensions of the TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4 nanocomposites. This effect was observed both in the absence and presence of 1,4-benzoquinone (Figures 1*B* and 1*D*), i.e., independent of the initial oxidative characteristics of the aqueous media. We assume possible mechanisms for enzymatic neutralization: 1) The enzyme proteins (via the variety of chemical groups) may act as radical scavengers, quenching ROS; 2) Components of the assay (NADH, FMN) might compete in redox cycling reactions, effectively "shunting" electrons away from the nanocomposite-driven pathways; 3) The proteins could chelate iron ions, sequestering them from participating in Fenton chemistry. The neutralization of ROS variations by bacterial enzymes was previously observed in suspensions of other magnetite-based nanocomposites (Supplementary Materials C, Figures S5C, S5D).

Moreover, consistent with our current observations in the enzyme solutions (Fig. 1), authors [20, 56] reported an alteration of ROS levels in a bacterial environment compared to non-biological aqueous media. This effect was attributed to the metabolic ability of bacteria to balance the external ROS level, adapting it to an optimum for bacterial physiology—a phenomenon known as a protective regulatory function of bacteria.

B. Effects of Nanocomposites TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4 on Bioluminescence Intensity of Enzyme System

We studied the efficiency of enzymatic process in the presence of the nanocomposites. Bioluminescence intensity was used as an indicator of the enzymatic efficiency. The experiments were carried out in the absence and presence of the model oxidizer 1,4-benzoquinone (Figures 2A, 2B). The results show that the values of I^{rel} were close to the control, indicating no significant effects of the nanocomposites on the enzymatic process.

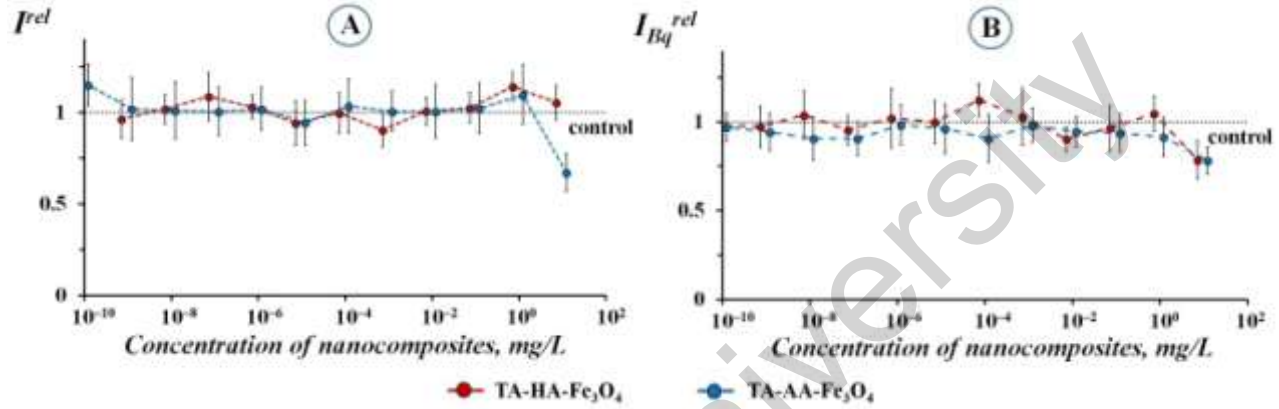

Figure 2. Relative bioluminescence intensity: (A) in the enzyme system, I^{rel};
(B) under conditions of oxidative stress in the enzyme system, I_{Bq}^{rel}. Control suspension — in the absence of nanocomposites. Conditions of oxidative stress — in the presence of 1,4-benzoquinone ($IC_{50Bq} = 10^{-6}$ M)

This result is likely related to the absence of nanocomposite effects on the ROS level in the enzymatic solutions (Figures 1*C* and 1*D*). As discussed previously [61–63], the bioluminescence oxidative reaction—involving its intermediate (peroxyhemiacetal)—and competing dark side reactions are sensitive to ROS levels.

Furthermore, the low sensitivity of the bioluminescence enzyme system to magnetite-based nanocomposites was demonstrated in prior work [20, 56] and Supplementary Materials D (Figures S6A, S6B), using nanocomposites such as Fe_3O_4-MOF, Fe_3O_4-HA, Fe_3O_4-APTES, Fe_3O_4-AA-MOF, and Fe_3O_4-HA-MOF as examples.

However, our previous studies have shown that triple nanocomposites of similar structure, specifically Fe_3O_4-AA-MOF [56] and Fe_3O_4-HA-MOF, did affect luminescent bacterial cells, demonstrating both toxicity and pro-oxidant activity. Furthermore, Fe_3O_4-AA-MOF retained the antibacterial properties of AA. Hence, the effects of these compounds on whole cells and isolated enzymes differed. This suggests that our current investigation should continue toward cellular processes, with special attention given to the role of the cellular membrane.

Conclusions

This study investigated the reactive oxygen species (ROS) generation and biological activity of silica-based nanocomposites, TA-AA-Fe_3O_4 and TA-HA-Fe_3O_4, in both non-biological (aqueous) and simple biological (enzymatic) systems. The key findings are as follows:

1. Neutralizing Effect of the Enzymatic System: The enzymatic bioluminescence system effectively neutralized the impact of the nanocomposites on ROS levels. In enzyme-free water, the nanocomposites caused significant and variable increases in ROS content (up to 300 % for TA-AA-Fe_3O_4, in the presence of the model oxidant 1,4-benzoquinone. However, in the enzymatic solution, these effects were abolished, and the ROS content remained stable and close to control levels, regardless of the presence of benzoquinone.

2. Reduced Data Variability in Biological Media: The presence of the enzymatic system significantly reduced the data spread and fluctuations in ROS measurements observed in non-biological aqueous suspen-

sions. The increase in the determination coefficients (R^2) quantitatively confirms a more predictable and stable behavior of the nanocomposites in the biological environment.

3. No Direct Effect on Enzyme Function: The nanocomposites did not exhibit any significant inhibitory or activating effects on the bioluminescence enzymatic process itself, as indicated by the stable relative bioluminescence intensity (I^{rel}). This suggests that the enzymes are not a primary target for these specific nanocomposites.

4. Divergence from Cellular Effects: Previous findings of bacterial toxicity and pro-oxidant activity suggest that the cellular context, particularly membrane interactions, is crucial for understanding nanoparticle bioactivity. The lack of effect on the isolated enzyme system contrasts with previous findings on bacterial cells, where similar nanocomposites (e.g., Fe_3O_4-AA-MOF) demonstrated toxicity and pro-oxidant activity. This highlights a critical difference: the cellular membrane and internal metabolic processes likely play a decisive role in the biological activity and toxicity of these nanocomposites, which is not captured by acellular enzymatic assays. Hence, our current investigation should continue toward cellular processes using cells of luminous bacteria as example.

In summary, while the silica-magnetite nanocomposites can change ROS level in simple aqueous environments, their activity is effectively modulated and neutralized in an enzymatic system. The results suggest that for an accurate prediction of *in vivo* biological activity, particularly for applications like ferroptosis-based therapy, further investigation must focus on cellular models where membrane interactions and complex metabolic pathways determine the final biological outcome.

Supporting Information

The Supporting Information is available free at https://ejc.buketov.edu.kz/index.php/ejc/article/view/500/308

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Arina Gennadievna Kicheeva** and **Ekaterina Sergeevna Sushko** investigation, formal analysis, visualization, writing — original draft, writing-review & editing; **Artur Albertovich Dzeranov** and **Lyubov Sergeevna Bondarenko** investigation, formal analysis, visualization, writing-review & editing; **Natalya Sergeevna Tropskaya** — validation, **Kamila Asylbekovna Kydralieva** and **Nadezhda Stepanovna Kudryasheva** conceptualization, formal analysis, project administration, supervision, validation, writing-original draft, writing-review & editing.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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