

Methodological aspects of small iron concentrations determination in black yeasts grown in the presence of iron oxide nanoparticles

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Abstract. Nonpathogenic *Exophiala nigrum* (black yeasts) unicellular organisms of the Baikal Lake were used as a model system for determination of small iron concentrations in the samples grown without or with controlled amount of maghemite nanoparticles (MNPs) in nutrient. MNPs were produced by the electrophysical laser target evaporation technique. Electrostatically stabilized suspensions were prepared using sodium citrate solutions in distilled water. We assumed that one maximum permissive dose of ionic iron in water 1 MPD is equal to 0.3 mg/L. For biological experiments Saburo liquid nutrient medium was prepared with iron concentrations of 0, 10², 10³ and 10⁴ MPD. One ml of *E. Nigrum* cell suspension was added to Saburo liquid nutrient for 24 hours exposure. Followed by sowing onto a solid agar Saburo for 30 days colonies grows. Biosamples for electron microscopy, magnetic and total reflection X-ray fluorescence spectroscopy measurements were collected simultaneously. We were able to comparatively analyze the trace concentrations of iron in the yeast of the order of 10 ppm for control group and 600 ppm for the group grown in the presence of 10⁴ MPD of iron.

1 Introduction

Living systems strongly depend on iron in variety of life steps [1-2]. Therefore, precise determination of a small amount of iron in the biological and artificial samples mimicking natural tissues is a challenge. Magnetic nanoparticles (MNPs) can enter occasionally or to be on-purpose injected for therapeutic action into the human body. Magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) are most studied magnetic biocompatible materials [3-4]. An accumulation of MNPs and their degradation products in the tissues/biofluids is a dangerous insufficiently understood health hazard [5]. Nonpathogenic *Exophiala nigrum* (unicellular black yeast) play an important role in the equilibrium of the Baykal Lake ecosystem [6]. *E. Nigrum* were previously used as a model system for the estimation of cytotoxicity, MNPs accumulation and inductive heat capacity studies of the samples grown in the presence of MNPs [4,7]. One of the main obstacles for rapid extension of biomedical applications of MNPs is the variation of the properties of MNPs from batch to batch [8]. Therefore, the fabrication techniques providing enhanced batches attract special attention. From this point of view electrophysical techniques of the electric explosion of wire ensuring fabrication of

spherical MNPs [9-10] and laser target evaporation (LTE) with the production rates up to 50 g/h [11-12] are of special interest. Preparation of model samples with of MNPs is highly requested nowadays for the development of cheap compact analytical devices (magnetic biosensors) [13-15]. They are also requested for better understanding of mechanisms and elaboration of the treatment conditions for such cancer therapies as hyperthermia and thermal ablation [4, 16-17].

In this work, maghemite spherical magnetic nanoparticles were fabricated by LTE technique, and water-based suspensions were prepared on the basis of obtained MNPs. Their physical properties were carefully checked by a number of techniques prior to determination of trace concentrations of iron in the *Exophiala nigrum* samples grown without or with controlled amount of MNPs in nutrient medium.

2 Experimental

Iron oxide MNPs were synthesized by LTE method using Ytterbium (Yb) fiber laser with 1.07 μm wavelength. More details can be found elsewhere [4, 7]. Structural studies of air-dry MNPs were performed by transmission electron microscopy (TEM) using a JEOL

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JEM2100 microscope operating at 200 kV and scanning electron microscopy (Hitachi S-4800, SEM). The X-ray diffraction (XRD) studies were performed by DISCOVER D8 (Bruker) diffractometer using Cu-K α radiation (wave lengths $\lambda=1.5418 \text{ \AA}$).

Electrostatically stabilized suspensions were prepared by ultrasound treatment using sodium citrate solutions (5 mM) in distilled water. De-aggregation was insured by ultrasound treatment on Cole-Parmer CPX-750 homogenizer operated at power output 300 W. During the ultrasound treatment the decrease of an average hydrodynamic diameter of aggregates in suspension was monitored by dynamic light scattering. A Hermle Z383 centrifuge operated at 10,000 rpm for 10 min was used to remove all large aggregates. The specific surface area of MNPs was measured by the low-temperature sorption of nitrogen (Brunauer–Emmett–Teller physical adsorption method, BET) using Micromeritics TriStar 3000 analyzer. More details on suspension preparation can be found elsewhere [4, 11].

Maximum permissible dose of iron in water (MPD) is determined as 0.3 mg/L for ionic iron [18]. The control was Saburo liquid medium with the corresponding addition of a sterile physiological solution without MNPs. As the first step of the biological experiments Saburo liquid nutrient medium was prepared with following iron concentrations (provided by MNPs from suspension): 10^2 , 10^3 and 10^4 MPD. One ml of *E. Nigrum* cell suspension was added to Saburo liquid nutrient for 24 hours exposure in flasks using a mechanical rocking chair. At the end of the exposure period sowing onto a solid agar Saburo was done from which biosamples for TEM, magnetic measurements and total reflection X-ray fluorescence (TXRF) measurements were collected simultaneously after 30 days colonies grows. For TEM studies of biological samples cell cultures were pre-fixed in 0.5% glutaraldehyde in Sörenson buffer and centrifuged. The supernatant was removed and 2% glutaraldehyde in Sörenson buffer was added to the pellet. Samples were fixed in 1% Osmium Tetroxide in Sörenson buffer, dehydrated, embedded in EponPolarbed resin and cut in ultrathin sections for TEM studies (Philips EM208S). More details on LTE technique can be found elsewhere [15]. TXRF is a method of elemental analysis that can be applied to samples in the form of thin layer. Nanohunter spectrometer by Rigaku and previously elaborated protocol [7] were used. For both scanning electron microscopy (SEM) and TEM of *E. Nigrum* cell cultures were prepared by standard procedure [15]. Magnetic measurements were carried out on a MPMS XL-7 SQUID-magnetometer in polystyrene envelopes of known mass, contribution of which was carefully subtracted from the biological sample signal. Magnetic measurements were done at room temperature.

3 Results and discussion

XRD spectra gave a mean crystallite size for air-dried MNPs of $19 \pm 2 \text{ nm}$ in a good agreement with TEM and specific surface area evaluation. Fig. 1 shows the air-dry



Fig. 1. LTE iron oxide MNPs, TEM (a). Petri dish with *E. Nigrum* grown onto solid nutrient medium without MNPs (b). General view of *E. Nigrum* grown in the presence of 0 (c) or 10^4 MPD (d) of iron: MPD of iron provided by MNPs.

spherical LTE MNPs. The experimental XRD data were well fitted by the magnetite database. The chemical composition of LTE MNPs ($Fe_{2.72}O_4$) close to the stoichiometric maghemite was determined by the combination of red-ox titration and the lattice period analysis provided by XRD as in [9]. We were not able to observe significant difference in the average size/shape of the cells in all cultures under consideration grown in the presence or absence of MNPs (Fig. 1-2, Table 1).

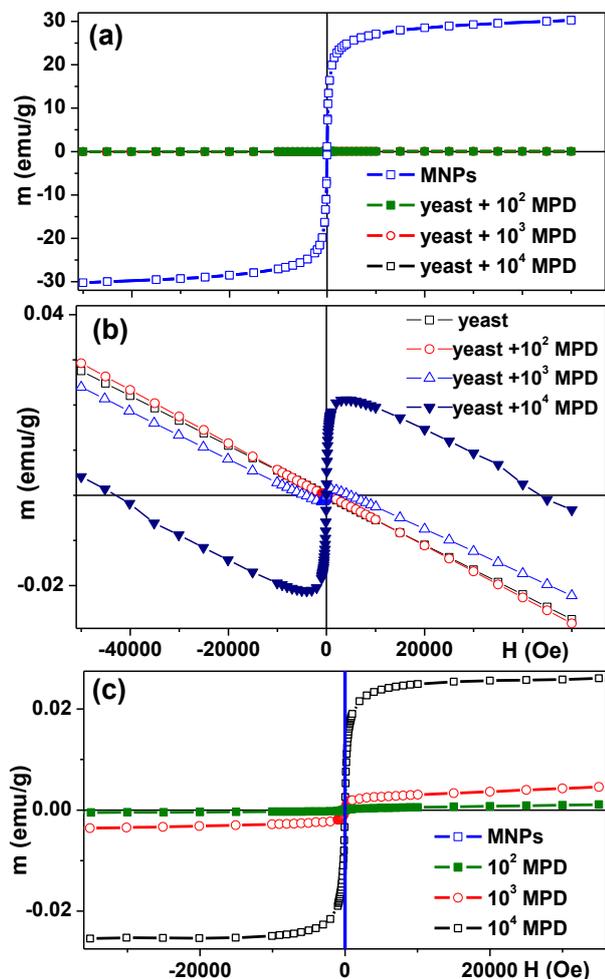


Fig. 2. Hysteresis loops of LTE MNPs from ferrofluid and *E. Nigrum* cell cultures grown in the presence of different amounts of MNPs (a). Hysteresis loops of all cell cultures (b). Recalculated hysteresis loops of all cell cultures grown in the presence of MNPs without contribution of yeast part (c).

Table 1. Iron concentration in *Exophiala Nigrum* cell cultures grown without or in the presence of MNPs. M_s^* - magnetic moment for 1 g of the biosample in the field of 35 kOe.

MPD in nutrient mg/L	M_s^* (emu/g) SQUID	ppm Fe by SQUID	ppm Fe by TXRF
0	0.0000	0 ± 10	9 ± 10
10 ²	0.0009	21 ± 10	15 ± 10
10 ³	0.0043	101 ± 20	44 ± 15
10 ⁴	0.0255	603 ± 100	440 ± 50

Fig.2(a) shows results of the SQUID measurements of the air dried MNPs and yeast samples. The yeast samples were taken by sterile scribe and immediately placed into polystyrene envelopes of known mass, mass

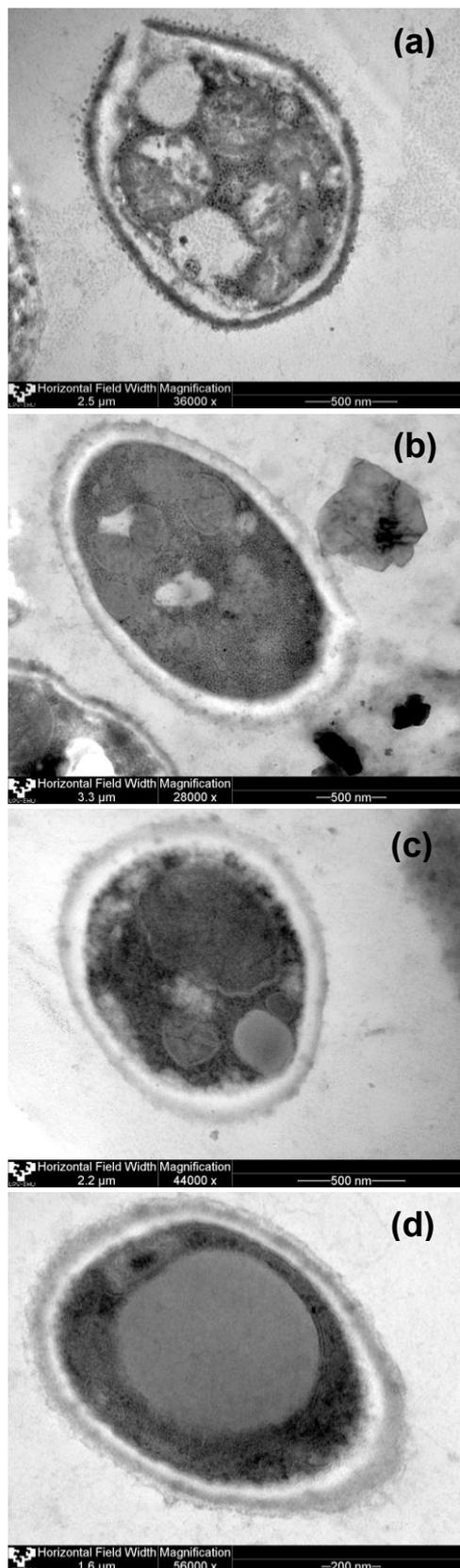


Fig. 3. TEM image of *E. Nigrum* cells grown without MNPs (a) and with 10² (b), 10³(c) and 10⁴ MPD of iron.

of the yeast was measured. The polystyrene contribution was previously measured by SQUID in the same conditions. As to expect, the highest magnetic moment corresponds to the MNPs dried from ferrofluid due to the

highest MNPs concentration. No saturation at room temperature was observed in the field of 50 kOe which is typical for superparamagnetic samples [4,14]. Observed magnetization value is consistent with the mean diameter of 19 ± 2 nm for MNPs [7, 9]. Although non-zero coercivity (≈ 50 Oe at 300 K) was observed it can be understood in the frame of the core-shell model invoking a ferromagnetic core and a spin-glass like surface layer in which the spins are frozen without long range magnetic order [11].

Magnetic moment (m) field dependences for all cases had strong diamagnetic contribution as to expect for water containing biosamples (Fig. 2(b)). In order to calculate the magnetic signal corresponding to MNPs per mass unit we used *E. Nigrum* cell culture grown without MNPs signal for calibration. For each field point we recalculated the hysteresis loops of all samples without diamagnetic contribution (Fig. 2(c)). As the saturation was not reached even for high field all calculations were made for the field of $H = 35$ kOe which was reasonably high: M_s^* is a magnetic moment of 1 g of biosample for the external field $H = 35$ kOe. Increase of the M_s^* value with MPD increase and “S” shapes of the hysteresis loops of biosamples after exposure to MNPs indicate MNPs accumulation with the increase of MPD of iron in the initial nutrient. Taking into account parameters of the hysteresis loop for MNPs we were able (Table 1) to recalculate iron concentration in ppm for SQUID measurements and compare them with ppm concentrations obtained by TXRF studies of dried samples: the higher MPD in nutrient the higher ppm of iron in the biological samples in both TXRF and SQUID measurements. For recalculation we considered that air-dry MNPs magnetic signal ($H = 35$ kOe) corresponds to 100 % of concentration. Therefore, simple proportion relations with air-dry signal as calibration gave numbers for all concentrations. One can see that SQUID measurements tend to give slightly higher value comparing with TXRF which is surprising because TXRF measures both ionic and MNPs contribution but SQUID signal is mostly corresponds to the MNPs. More studies are necessary in order to clarify the origin of such a discrepancy but it seems to be that *E. Nigrum* cells are rather large and special improved protocol is necessary for thin film on their basis preparation for TXRF. Trace iron concentrations indicated limited penetration of MNPs inside the cell or their adsorption by cell membranes (Fig. 3 shows some examples). One can clearly see thick cell membrane with typical for *E. Nigrum* morphology. Comparative estimation of the average size of the oval-shaped *Exophiala Nigrum* cells gives approximate values of $6 \pm 1 \mu\text{m} \times 3 \pm 1 \mu\text{m}$ for SEM and of $5 \pm 1 \mu\text{m} \times 3 \pm 1 \mu\text{m}$ for TEM studies for all cell cultures in a good agreement with each other.

4 Conclusions

Nonpathogenic *E. Nigrum* unicellular yeasts were used as a model system for determination of trace iron concentrations. They were grown in the presence of 19 ± 2 nm maghemite MNPs produced by laser target

evaporation. Electrostatically stabilized water based suspensions were prepared: liquid nutrient medium was elaborated with different iron concentrations of 0 to 10^4 MPD provided by MNPs. One ml of *E. Nigrum* cell suspension was added to Saburo liquid nutrient for 24 hours exposure followed by sowing onto a solid agar Saburo for 30 days cell grows after which the biosamples for TEM, magnetic and TXRF measurements were collected simultaneously. In both TXRF and SQUID measurements similar tendency was observed: the higher MDP in nutrient the higher ppm of iron in the biological samples. Trace iron concentrations of the order of 10 ppm for control group and 600 ppm for the group grown in the presence of 10^4 MPD of iron provided by MNPs indicated limited penetration of MNPs into the cell or their adsorption by membranes.

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