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Synthesis, physical characterization, antifungal and antibacterial activity of oleic acid-capped nanomagnetite and cobalt-doped nanomagnetite

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Abstract

Nanoparticles, 10-14 nm, consisting of either Fe₃O₄ or Co₀.₂Fe₂.₈O₄ stabilized with oleic acid, were prepared using solution combustion. Their structural and magnetic properties were examined using X-ray diffractometry, scanning electron microscopy, vibrating sample magnetometry, and Fourier-transform infrared spectroscopy. The properties of both sets of materials are similar except the cobalt-doped particles are considerably less magnetic. The *in vitro* inhibitory activities of the nanoparticles were assessed against pathogenic bacteria *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Streptococcus pyogenes*, and pathogenic fungi and molds *Candida albicans*, *Fusarium oxysporum* and *Aspergillus fumigatus*. The magnetite nanoparticles were moderately effective against all tested pathogens, but the activity of the cobalt-doped nanoparticles was significantly lower, possibly due to an interruption of the Fenton reaction at the bacterial membrane. This work suggests that potentially doping magnetite with stronger metal oxidants may instead enhance their antimicrobial effects.

**Keywords:** Magnetic nanoparticles, cobalt-doped magnetite, antifungal activity, antibiotic.
1. Introduction

Nanomagnetite, Fe$_3$O$_4$ formulated as a nanoparticle, has been used for a variety of biomedical applications including for biosensors,\textsuperscript{1} drug delivery,\textsuperscript{2-3} hyperthermic therapy,\textsuperscript{4} magnetic resonance imaging,\textsuperscript{5-7} and medical diagnostics and therapy.\textsuperscript{8-11} It is a promising biomedical material due to its high degree of chemical stability, magnetic behaviour, and biocompatibility.\textsuperscript{12-19} The physical and magnetic properties can be further tuned through controlled doping with other metals. Cobalt-doping magnetite provides the materials with increased hardness, higher electrical resistivity and higher electrical permeability at higher frequencies.\textsuperscript{20-21}

Our previous work with nanomagnetite has focused on using them as antibiotics,\textsuperscript{22-26} and there are multiple excellent recent reviews on the subject.\textsuperscript{27-28} Nanomagnetite has been extensively studied by others for antibiotic applications as core-shell formulations,\textsuperscript{29-30} as uncoated nanoparticles,\textsuperscript{31-32} as nanoparticles either doped or combined with other metals,\textsuperscript{33-35} or simply as drug delivery vehicles where the antibiotics adsorbed onto the surface.\textsuperscript{36} We have previously investigated the antibiotic potential of uncoated magnetite prepared using an additive-free electrochemical approach.\textsuperscript{23-26} The surface of these particles incorporated highly oxidized impurities that both inhibited aggregation and were responsible for the potent antibacterial activity. However, we wanted to explore the activity of a more traditional magnetite formulation and help determine whether the activity observed was due to the presence of Fe-O-O-H groups or due to the activity of the magnetite functionality. Doping with different metals might affect the behaviour of the materials; for example, Zn-doped nanomagnetite showed greater activity (defined in terms of inhibition zone diameter) than Fe$_3$O$_4$ alone.\textsuperscript{37} This activity was ascribed to the increased specific surface area. However, the antibiotic activity of cobalt-doped magnetite has not been extensively studied and the little recent research has largely been restricted to antibacterial behaviour,\textsuperscript{38-41} although there are
some notable exceptions: Žalnėravičius and co-workers showed that nanomagnetites with varying cobalt content and capped with L-lysine were potent agents against *E. coli*, *S. aureus*, and the fungi *C. parapsilosis* and *C. albicans*, and that activity was highly dependent on nanoparticle size and cobalt content. Smaller particles, and less cobalt content was associated with more potent activity.

Antifungal function might prove highly attractive for many consumer products to reduce molds and fungal biofilms. We know that our previously generated nanomagnetites showed very little toxicity towards mammalian cells while being highly toxic to both Gram-negative and Gram-positive bacteria. This selective activity was likely due to the difference in biofilm formation around the nanoparticles, and it is unclear what the activity would be against a eukaryotic fungus.

For this study we are studying the activity against *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Streptococcus pyogenes*, and pathogenic fungi and molds *Candida albicans*, *Fusarium oxysporum* and *Aspergillus fumigatus*. These are all high-risk pathogens. *S. dysenteriae*, as implied by the name, releases the Shiga toxins that cause gastroenteritis and can lead to severe complications including renal failure and haemorrhagic colitis. *K. pneumoniae* is a common bacterium previously associated with community-acquired pneumonia, but whose main feature of interest is as the source of carbapenem resistant genes that are spreading to other bacteria and contributing to the antibiotic resistance threat. *A. baumannii* strains resistant to all known antibiotics have been identified and the pathogen is a leading cause of hospital acquired pneumonia and can readily lead to death in already compromised patients. *S. pyogenes*, group A *Streptococcus*, is responsible for many cases of necrotizing fasciitis and is responsible for 160,000 deaths globally each year often through rheumatic fever; fortunately it is still largely susceptible to antibiotic treatment. *C. albicans* is one of the best studied fungal pathogens as it is a near-universally present member
of a healthy human oral microbiome, but one that can, for immunocompromised individuals, cause inflammatory oral candidiasis.\textsuperscript{50} It is also the pathogen largely responsible for vulvovaginal “yeast infections” and, if it enters the blood stream, it can often lead to fatal infection. \textit{F. oxysporum} is mainly of interest as a plant pathogen and is responsible for “banana wilt” which is threatening the genetically monodisperse Cavendish banana, the variety most familiar with consumers. There are no effective countermeasures available.\textsuperscript{51} \textit{A. fumigatus} is a ubiquitous and extremely thermotolerant mold that emerged as a leading cause of opportunistic fungal infection in humans that has been partially tamed through the use of azole antifungals. Unfortunately, azole-resistant strains have started rapidly spreading around the world in recent years.\textsuperscript{52} Together these pathogens threaten human and agricultural health, and many are at the forefront of the antibiotic resistance phenomenon and new classes of therapeutic interventions are required.

Here we report our investigations into the synthesis of nanomagnetite and cobalt-doped nanomagnetite terminated with oleic acid, a common terminating agent,\textsuperscript{53-54} and their physical, magnetic, and biological characterization including their activity against these pathogenic bacteria and fungi.

\section*{2. Experimental}

\subsection*{2.1. Materials and General Methods}

Oleic acid hydrate, cobalt nitrate hexahydrate, iron (III) nitrate nonahydrate, iron (II) chloride tetrahydrate, toluene, and sodium hydroxide were purchased from Millipore Sigma and used as received. For the \textit{in vitro} analysis the positive controls ampicillin, gentamicin, terbinafine and canazole were purchased from Millipore Sigma and used as received. Fungal and bacterial culture media including Roswell Park Memorial Institute 1640 (RPMI 1640) medium buffered to pH 7.0 with morpholine propane sulfonic acid (MOPS); Mueller-Hinton
broth (MHB) and Mueller-Hinton agar (MHA), were obtained from HiMedia (Mumbai, India).

Gram-negative bacterial strains *Shigella dysenteriae* (PTCC 1188), *Klebsiella pneumoniae* (PTCC 1290), *Acinetobacter baumannii* (PTCC 1855); Gram-positive *Streptococcus pyogenes* (PTCC 1447); pathogenic yeast *Candida albicans* (PTCC 5027); and molds *Fusarium oxysporum* (PTCC 5115) and *Aspergillus fumigatus* (PTCC 5009) were obtained from the Persian Type Culture Collection (Karaj, Iran).

### 2.2. Synthesis of the Fe$_3$O$_4$ and Co/Fe$_3$O$_4$ nanoparticles

The oleic acid-capped Fe$_3$O$_4$ and Co-doped Fe$_3$O$_4$ nanoparticles were prepared using chemical co-precipitation and thermal combustion similar to previously published approaches.$^{54-55}$ An aqueous solution was prepared by dissolving iron (II) chloride tetrahydrate (1.00 g, 7.89 mmol) and iron (III) nitrate nonahydrate (5.30 g, 21.9 mmol) in a 1:2 molar ratio in 30 ml of distilled water already containing toluene (40 mL) and oleic acid (1.30 g, 4.60 mmol). The mixture was magnetically stirred at 70 °C to initiate the solution combustion$^{56}$ while 4 mL of 25% aqueous ammonia was added in one batch to the solution to increase the pH to 10.5. The mixture is allowed to continue stirring while the reaction occurs. WARNING: Extremely exothermic reaction occurs. The resulting black precipitate was collected by filtration and extensively washed with deionized water; with the material centrifuged at 10000 rpm and the supernatant decanted between each wash, before being dried at 70 °C for 2 h.

The cobalt-doped iron oxide nanoparticles (Co/Fe$_3$O$_4$) were prepared in a similar fashion by introducing a controlled amount of cobalt nitrate into the initial solution. In a typical procedure, 0.43 g of Co(NO$_3$)$_2$·(H$_2$O)$_6$ was added to iron (II) chloride tetrahydrate (0.56 g) and iron (III) nitrate nonahydrate (5.26 g) in a 1:2 molar ratio in 30 ml of distilled water already containing toluene and oleic acid as described above. The solution was then treated identically to the solution above to provide Co$_{0.2}$Fe$_{2.8}$O$_4$. 

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2.3. Characterization of the Fe₃O₄ and Co₀.₂Fe₂.₈O₄ nanoparticles

X-ray diffraction (XRD) characterization was conducted on an X’pert Pro MPD (Malvern) X-ray diffractometer equipped with a Cu Kα radiation source. The morphology of the samples was studied using a scanning electron microscope (SEM) and the EDXS spectra and atomic quantification were acquired at the same time (KYKY-EM3900M, Beijing China). Vibrating sample magnetization (VSM) was carried out using an MDKB VSM instrument (Danesh Pajouh Company, Kashan, Iran). FTIR spectroscopy of the nano-structures was conducted by first suspending them in a KBr pellet and then using a 460 PLUS Jasco spectrometer scanning from 400 to 4000 cm⁻¹. All experiments were conducted at ambient temperature (23-25 ºC).

2.4. In vitro inhibitory activities of nanoparticles

Broth microdilution and time-kill methods were applied to assay antimicrobial susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) guidelines M07-A9,⁵⁷ M27-A2,⁵⁸ M38-A2,⁵⁹ and M26-A.⁶⁰ The results were the average of three independent experiments. For these experiments, the yeast, mold and bacterial suspensions were prepared in the appropriate broth media (as indicated above) at 0.5-2.5 × 10³, 0.4-5 × 10⁴ and 5 × 10⁵ CFU ml⁻¹ respectively.⁶¹

2.4.1. MIC testing

Aliquots of the nanoparticle solutions, 20 μL at a concentration of 20,480 μg ml⁻¹ in distilled water, were added to both the first and second wells in each row of a 96-well microliter plate. 20 μl distilled water was added to wells 2-12, and two-fold serial dilutions were carried out in them by transferring 20 μl from the previous well (making the total temporarily 40 μl), mixing thoroughly with the pipette, and adding 20 μl to the next well; for the final well, 20 μl was withdrawn and discarded after mixing. 80 μl of MHB (for bacteria) or RPMI 1640 (for fungi) with 100 μl of the prepared microbial suspensions (see above) were then added to all the wells. This provides, a concentration range of 2048-1 μg ml⁻¹ of each derivative in each row. These
microliter plates were incubated with shaking at 100 rpm at 37 °C for 24 h. The lowest concentration of derivatives that resulted in no visible turbidity was considered the MIC value. Experiments were repeated on two additional separate occasions with fresh preparations of pathogens. Results from the three experiments were identical.

2.4.2. MBC and MFC testing

Samples of all wells that showed no growth in the MIC test, were cultured in MHA or RPMI 1640 agar, which then were incubated at 37 °C for another 24 h. The MBC and MFC was identified as the lowest concentration at which no microbial populations were present.

3. Results and Discussions

3.1. XRD characterization

The nanoparticles were prepared by mixing Fe (II), and Fe (III) with or without Co (II) in aqueous solution and conducting solution combustion. Under these conditions, the cobalt is oxidized to cobalt (III) during the process and these tetrahedral (as opposed to octahedral Co (II)) are incorporated into the lattice. The resulting particles were characterized by XRD (Figure 1). The spectra are consistent with the reported JCPDS spectra for both samples (JCPDS 003-0863) with Bragg peaks of 30.4° (2 2 0), 35.8° (3 1 1), 43.4° (4 0 0), 53.5° (4 2 2), 57.2° (5 1 1), 63.2° (4 4 0) and 74.2° (5 3 3).
1182 Figure 1. XRD spectra of Fe₃O₄ (A) and Co₀.₂Fe₂.₈O₄ (B) nanoparticles recorded at 23 °C.

The average crystallite size of the nanostructures was calculated from peak (3 1 1) using the Sherrer formula.³²,⁶⁴

\[ D_{h,k,l} = \frac{0.9 \lambda}{(\beta_{h,k,l}\cos\theta)} \]  

Where \( \lambda \) is the wavelength (\( \lambda = 1.542 \) Å (CuKα)), \( \beta \) is the product of the full width at half maximum (FWHM) of the selected peak and \( \pi/2 \) as it approximates a Gaussian distribution. \( \theta \) is the diffraction angle of the peak.

The average crystallite sizes for the nanomagenetite and cobalt-doped nanomagnetite were 14 and 10 nm respectively. The XRD spectra for both samples were similar and could not be used to confirm the presence of cobalt in the crystal.

3.2. Morphological Analysis

SEM was used to support the sizing of the materials (Figure 2), and showed that the structures formed (white spheres on a black matrix background) are roughly spherical and under 100 nm, although the aggregation behaviour under the SEM imaging conditions makes it challenging
to visualize individual particles. Unlike XRD, the energy dispersive X-ray analysis shows clear
evidence for the presence of both the cobalt and the iron in the samples and can be used to
quantify the relative atomic quantities of the species in the sample using external standards. This method provides the experimental formula of $\text{Co}_{0.21}\text{Fe}_{2.51}\text{O}_{4.28}$ for the batch used for the biological analyses. This is in reasonable agreement with the theoretical formula.

![Figure 2](image.png)

**Figure 2.** A) A representative SEM image, and B) the EDXS spectrum of the $\text{Co}_{0.2}\text{Fe}_{2.8}\text{O}_4$ nanoparticles.

### 3.3. FTIR Characterization

The FTIR spectra of both samples are provided as Figure 3.
Figure 3. FT-IR spectrum of Fe$_3$O$_4$ and Co/Fe$_3$O$_4$ nanoparticles

The spectra are largely identical as expected: broad peaks at 3600-3400 cm$^{-1}$ arise from O-H stretching of adsorbed water molecules. The low wavenumber cluster (500-600 cm$^{-1}$) are expected from the metal-oxygen vibrations, and the strong signal at 1390 cm$^{-1}$ is due to the stretching vibrations in adsorbed nitrate. Vibrations at 1624 cm$^{-1}$ correspond to the vibrations of the C-O of the oleic acid,$^{37}$ and the lack of a strong band at 1710 cm$^{-1}$ is consistent with an oleic acid monolayer.$^{53}$

3.4. Magnetic Measurements

Vibration sample magnetization was used to understand the magnetic behaviour of the materials. They are largely similar under all other characterization techniques, but the doping does have a significant impact on the magnitude of their magnetic behaviour (Figure 4, Table 1).
Figure 4. The magnetization loops of Fe$_3$O$_4$ and Co-doped Fe$_3$O$_4$ nanoparticles, recorded at 23 °C.

Table 1. Effect of Co on magnetic properties of the Fe$_3$O$_4$ NPs

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>$M_s$ (emu/gr)</th>
<th>$M_r$ (emu/gr)</th>
<th>$H_C$ (Oe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$_3$O$_4$</td>
<td>44.5</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Co-doped Fe$_3$O$_4$</td>
<td>19.0</td>
<td>1.9</td>
<td>19.3</td>
</tr>
</tbody>
</table>

VSM analysis confirms that the samples are superparamagnetic as expected. The differences in saturation magnetization ($M_s$), coercivity ($H_C$), and remnant magnetism ($M_r$) can be explained based on F-center exchange coupling (FCE) theory. Co-doped nanoparticles are more strongly affected by FCE interactions due to the smaller distance between the Co and the Fe ions. This traps electrons in the oxygen vacancy, which acts as a coupling center, and as a result increases the magnetization of the nanoparticles. The magnetization is affected as a consequence of Co concentration within the nano-structure. The small distances between Co
and iron ions are smaller than between iron atoms, and this can lead to trapping an electron in oxygen vacancy, which acts as a coupling center. This results in a change in the magnetization of the nanoparticles as a function of Co content.

### 3.5. Evaluation of antimicrobial activity

The inhibitory potential of nanoparticles was studied against both Gram-negative and Gram-positive bacterial strains as well as some fungal pathogens. Experiments were carried out in solution by using serial dilutions of stock solutions of the nanoparticles added to media inoculated with the pathogen at the appropriate concentration. The results were reported as the minimum inhibitory concentration (MIC) defined as the concentration at which no further increase in solution optical density was observed, the minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) defined as the concentration at which cell culture on appropriate petri dishes showed no growth (Tables 2 and 3).

### Table 2. Antibacterial activity of nanoparticles

<table>
<thead>
<tr>
<th>NPs</th>
<th>Antibiotics</th>
<th>Bacteria</th>
<th>MIC (μg ml⁻¹)</th>
<th>MBC (μg ml⁻¹)</th>
<th>Ampicillin</th>
<th>Gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₂O₃</td>
<td></td>
<td>Shigella &lt;em&gt;dysenteriae&lt;/em&gt;</td>
<td>512</td>
<td>&gt;2048</td>
<td>256</td>
<td>0.031</td>
</tr>
<tr>
<td>Co₀.₂Fe₂.₈O₄</td>
<td></td>
<td>Klebsiella &lt;em&gt;pneumoniae&lt;/em&gt;</td>
<td>512</td>
<td>&gt;2048</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acinetobacter &lt;em&gt;baumannii&lt;/em&gt;</td>
<td>512</td>
<td>&gt;2048</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Streptococcus &lt;em&gt;pyogenes&lt;/em&gt;</td>
<td>1024</td>
<td>&gt;2048</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

MIC (μg ml⁻¹), MBC (μg ml⁻¹)

### Table 3. Antifungal activity of nanoparticles

<table>
<thead>
<tr>
<th>NPs</th>
<th>Antifungals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₂O₃</td>
<td></td>
</tr>
<tr>
<td>Co₀.₂Fe₂.₈O₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fungi | Fe\textsubscript{3}O\textsubscript{4} | Co-Fe\textsubscript{3}O\textsubscript{4} | Terbinafine | Canazole
---|---|---|---|---
*Candida albicans* | MIC 2 | 1024 | 32 | 256
MFC 4 | 1024 | 64 | 512
*Fusarium oxysporum* | MIC 128 | >2048 | 32 | 256
MFC 256 | >2048 | 64 | 512
*Aspergillus fumigatus* | MIC 2 | 256 | 32 | 32
MFC 1 | 256 | 32 | 32

MIC (\(\mu g\) ml\(^{-1}\)), MFC (\(\mu g\) ml\(^{-1}\))

The unmodified magnetite nanoparticles efficiently blocked the growth of all bacterial and fungal pathogens; however, these oleic acid-capped compounds were not as effective as our previously reported uncapped, surfactant-free particles which showed MICs of 2.0 \(\mu g/ml\) against *S. aureus* and *E. coli*.\(^{25}\) The Co-doped Fe\textsubscript{3}O\textsubscript{4} nanoparticles showed no antibacterial activity. This difference in activity could be ascribed to the mechanism of action of these capped nanomagnetites compared to our previous systems.

The particles bind to the plasma membrane of the pathogens causing additional membrane disruption and ensuring that the generated reactive oxidative species are already co-localized to the lipids.\(^{28}\) The generally accepted mechanism of action for the antibacterial activity of capped-magnetite is through the conversion of endogenous hydrogen peroxide into more reactive oxygen species (ROS, superoxide, hydroxyl radical, proxy radical) that readily cause cellular damage through non-specific oxidation of the lipid membrane.\(^{59-70}\) This occurs through the slow oxidation of the magnetite (Fe\textsubscript{3}O\textsubscript{4}), which contains a mixture of Fe\textsuperscript{2+} and Fe\textsuperscript{3+} ions, to maghemite (\(\gamma\)-Fe\textsubscript{2}O\textsubscript{3}) through the Fenton reaction as the Fe (II) atoms slowly oxidize to the more stable Fe (III) (Figure 5).\(^{71}\) However, this may become more complicated in the presence of the cobalt (III). As the electron is released from the iron atom it could be trapped by the Co (III) to revert it to the highly stable Co (II). This would prevent the formation of the
highly oxidized species. Further investigations are required to explore and confirm this mechanism of action.

**Figure 5.** Schematic of the mechanism of action of the iron oxide nanoparticles, and a proposed mode of action for the cobalt-doped nanomagnetite as a possible cause of the lack of antibacterial activity of the cobalt-doped nanoparticles.

In contrast, the magnetite was observed to be quite a potent antifungal with lower MICs and MFCs lower than front-line antifungals terbinafine and canazole.\textsuperscript{72-73} The cobalt-doped materials show some slight activity. To contextualize these results, the activity is considerably better than that observed by Seddighi and their larger iron oxide nanoparticles.\textsuperscript{31} They observed MFCs of between 500-1000 μg/mL for particles 30-40 nm in diameter (ours are closer to 14 nm). The smaller particles are expected to be more effective as the cytotoxic ROS production is a function of surface area. Anghel and co-workers used similar oleic acid-coated magnetite nanoparticles to inhibit fungal growth on textiles, but do not report the size of the particles to allow for direct comparison.\textsuperscript{44} Žalnėravičius and co-workers carried out two studies using cobalt-doped magnetite as antifungal agents. However, they did not compare the efficacy of the particles against antifungals and report the exclusion diameter rather than an MIC and so it...
is hard to compare the results to the current study.\textsuperscript{42-43} In their work, smaller particles were found to be more active, magnetite was found to be more active than cobalt-doped magnetite, and antimicrobial activity decreased as cobalt content increased. This is consistent with our current results and is possibly explainable by the decreased production of ROS. Doping the magnetite with stronger oxidants than Fe (II) might invert this attenuation of activity. Regardless, this magnetite is considerably less active against bacteria compared to our previously prepared uncapped magnetite which has not been evaluated against fungi to date; unsurprisingly, masking the surface of the metal nanoparticle decreases their activity.

6. Conclusion

Nanomagnetite (Fe$_3$O$_4$) and Co-doped nanomagnetite (Co$_{0.2}$Fe$_{2.8}$O$_4$) stabilized with oleic acid were synthesized via co-precipitation with diameters of ~10–14 nm. The two sets of materials showed similar physical characterization, but the cobalt-doped materials were considerably less magnetic. They also differed greatly in biological activity: the oleic acid-terminated nanomagnetite is a potent antibacterial and very potent antifungal. Introducing cobalt greatly decreases their antibiotic activity. The introduction of stronger metal oxidants than Fe (II) such as copper, tin, chrome, zinc and magnesium may improve their antimicrobial effects.

Acknowledgments

A. Rahdar would like to thank the University of Zabol for financial support (UOZ-GR-9618-40) for this work. JFT gratefully acknowledges financial support from the University of Windsor (JFT grant no 817074), the Natural Sciences and Engineering Research Council of Canada (JFT grant no 2018-06338). The authors declare no competing financial interests.

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