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Synthesis of Fe_3O_4/Graphene/TiO_2 Composites for the Highly Selective Enrichment of Phosphopeptides from Biological Samples

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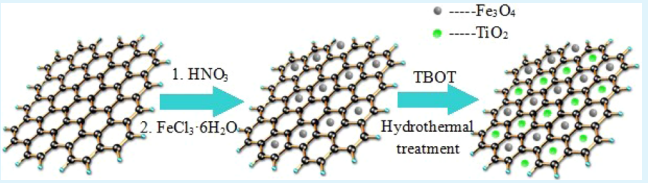
Supporting Information

ABSTRACT: In this work, Fe_3O_4/graphene/TiO_2 composites with a large surface area were designed and synthesized for the selective extraction and enrichment of phosphopeptides from biological samples. First, magnetic graphene was prepared according to our previous method. Next, we made the Fe_3O_4/graphene/TiO_2 composite precursor using tetrabutyl titanate. Fe_3O_4/graphene/TiO_2 composites were obtained after solvothermal and calcination treatments. We used standard protein-digestion solutions and human liver samples to test the enrichment ability of the obtained Fe_3O_4/graphene/TiO_2 composites. The experimental results demonstrate that Fe_3O_4/graphene/TiO_2 composites have a good phosphopeptide enrichment ability.



KEYWORDS: *Fe_3O_4/graphene/TiO_2 composites, enrichment, phosphoproteomics, mass spectrometry*

1. INTRODUCTION

Phosphorylation is one of the most common post-translational modifications of proteins. It regulates cellular activities including signal transduction, cell-cycle control, proliferation, differentiation, transformation, and metabolism. As such, protein phosphorylation has relevance to the regulation of biological pathways in cancer cells.[1−5] Studies of protein phosphorylation may help to understand better both cell-signaling pathways and the molecular classification of disease. In addition, the analysis of novel kinase inhibitors may have a great importance for the study of diseases and is also helpful for finding new drug targets.[6−10] Over the years, the significance of protein phosphorylation has lead to many efforts to analyze protein phosphorylation. As we know, mass spectrometry is the most powerful tool for the study of phosphoproteins. However, it is difficult to detect them because phosphopetides exist in small quantities with low stoichiometries. To overcome the problems in phosphopeptide analysis, many different kinds of techniques and methods have been developed for the extraction and enrichment of phosphopeptides. Immunoprecipitation, metal-oxide-affinity chromatography, immobilized metal-affinity chromatography, and strong cation-exchange chromatography[11−17] are all strategies studied by researchers. As we know, scientists have demonstrated that the techniques of immobilized metal-affinity chromatography and metal-oxide-affinity chromatography are the most useful tools in phosphoprotein research.

Recently, graphene has attracted lots of interest because of its outstanding mechanical, electrical, thermal, and optical properties as well as its theoretically high surface area of 2600 $m^2\ g^{-1}$.[18−21] These outstanding features make graphene promising for many different kinds of potential applications, including nanoelectronics, batteries, and so forth.[22−25] Because of its outstanding features and tendency to aggregate when its dispersion solutions are dried, graphene as a support, decorated with metal nanoparticles and metal-oxide nanospheres, has recently been reported.[26−30] The superparamagnetism and biocompatibility of iron-oxide materials[31,32] as well as the commendable specific interactions of titania with the phosphate groups of phosphopeptides[33−35] lead us to synthesize a kind of nanomaterial that can gather all of the advantages of graphene, iron oxide, and titania, leading to its excellent enrichment efficiency for phosphopeptides.

Here, we designed and synthesized Fe_3O_4/graphene/TiO_2 composites that have a large specific surface area, and we applied them to the selective enrichment of phosphopeptides. The novel materials were evaluated using protein digestion. We found that they have both enrichment efficiency and enrichment selectivity. In addition, we applied them to the enrichment of phosphopeptides from lysates of human liver cancer tissue from a liver cancer patient; more than 200 phosphopeptides were found, further proving the effectiveness of this method.

2. EXPERIMENTAL SECTION

2.1. Synthesis of Fe_3O_4/Graphene/TiO_2 Composites. The synthesis approach for Fe_3O_4/graphene/TiO_2 composites is shown in Scheme 1. At first, magnetic graphene was synthesized according to previous methods.[36,37] Briefly, 400 mg of graphene was dispersed into 50 mL of concerntrated nitric acid at 60 °C with magnetic stirring for 7 h. The graphene treated by HNO_3 was collected by washing with water five times and dried under vacuum at 50 °C. The dried, pretreated graphene (150 mg) and $FeCl_3{\cdot}6H_2O$ (405 mg) were dispersed into 40 mL of ethylene glycol solution with trisodium citrate (0.15 g), sodium acetate (1.8 g), and poly(ethylene glycol)-20 000 (1.0

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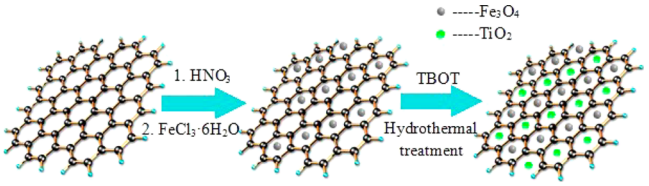
Scheme 1. Schematic Diagram of the Synthetic Route of Fe_3O_4/Graphene/TiO_2 Composites



g) by ultrasonication and magnetic stirring for 2 h. The mixture was sealed in the autoclave and heated at 200 °C for 10 h. Finally, the obtained magnetic graphene was washed with water and collected by magnetic-separation techniques. Next, we synthesized the Fe_3O_4/graphene/TiO_2 composite precursor. The detailed steps are as follows: 0.15 g of the obtained Fe_3O_4/graphene composites were dispersed in 200 mL of absolute ethanol followed by adding a concentrated ammonia solution (0.9 mL, 28 wt %) and treating with ultrasound for 15 min. Tetrabutyl titanate (TBOT, 2.0 mL) was added over 5 min, and the hydrolysis and condensation reaction was performed (24 h, 45 °C). After washing with deionized water and ethanol, 0.5 g of the Fe_3O_4/graphene/TiO_2 composite precursor and 20 mL of deionized water were mixed. The mixture was transferred into an autoclave (30 mL), and the reaction was performed at 160 °C for 24 h. After cooling to 25 °C, the resultant products were collected, washed, dried, and calcined according to our previous method.[35] The obtained products were used in the subsequent work.

2.2. Selective Capture of Phosphopeptides from Protein Digestion and Human Liver. After digestion, the peptides from standard proteins were first diluted according to a previous method.[35] Next, a suspension of Fe_3O_4/graphene/TiO_2 composites (400 μg) was added into 200 μL of the peptide solution. The enrichment was performed at 25 °C for 30 min. The peptides adsorbed on Fe_3O_4/graphene/TiO_2 composites were magnetically separated. The peptides captured by Fe_3O_4/graphene/TiO_2 composites were washed and eluted according to our previous method.[35] Finally, the eluate was analyzed by MALDI-TOF MS.

According to the previous method,[35] the human liver sample was prepared and digested. After digestion, the phosphopetides in the real sample were selectively enriched using Fe_3O_4/graphene/TiO_2 composites. After elution, the phosphopeptides were analyzed by LC–ESI MS.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Fe_3O_4/Graphene/TiO_2 Composites. Scheme 1 shows the synthesis approach for the Fe_3O_4/graphene/TiO_2 composites.[37] At first, the magnetic graphene was prepared according to a previous method. Next, the Fe_3O_4/graphene/TiO_2 composite precursor was synthesized via TBOT hydrolysis, and the Fe_3O_4/graphene/TiO_2 composites were obtained after solvothermal and calcination treatments.

We used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to characterize our obtained products. The results in Figure 1 show that the graphene sheets have a nearly transparent flakelike shape with characteristic crumpled silk waves[38] and single-layer nature. After modification with Fe_3O_4 and TiO_2 before solvothermal treatment, magnetite beads were seen on the surface of graphene sheets (Figure 1a), but TiO_2 was not so clear. The comparison of panels a and b in Figure 1 showed that lots of smaller spheres were observed after solvothermal and calcination treatments, which indicate the successful combination of TiO_2 on the surface of magnetic graphene. Using the proposed method, amorphous TiO_2 transformed to crystallized TiO_2 nanoparticles. As seen in Figure 2, this was also demonstrated using wide-angle X-ray diffraction.

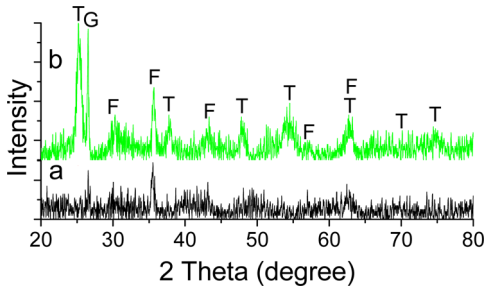


Figure 2. Wide-angle XRD patterns of (a) the Fe_3O_4/graphene/TiO_2 composite precursor and (b) Fe_3O_4/graphene/TiO_2 composites after hydrothermal and calcination treatments.

The energy-dispersive X-ray analysis (Figure S2) shows the existence of C, O, Fe, Ti, and Cu elements, further demonstrating the successful coating of Fe_3O_4 and TiO_2. In addition, we measured the content of C, H, and O in the graphene oxide and the thermally treated graphene oxide via elemental analysis and found that the oxygen content is almost the same in the both samples, indicating a relatively good thermal stability of the graphene oxides.

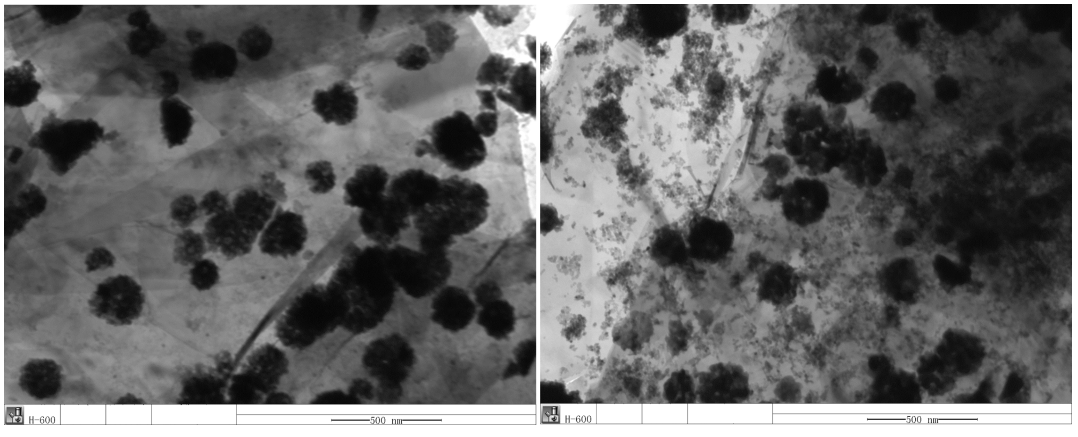


Figure 1. TEM of (a) the Fe_3O_4/graphene/TiO_2 composite precursor and (b) Fe_3O_4/graphene/TiO_2 composites after hydrothermal and calcination treatments.

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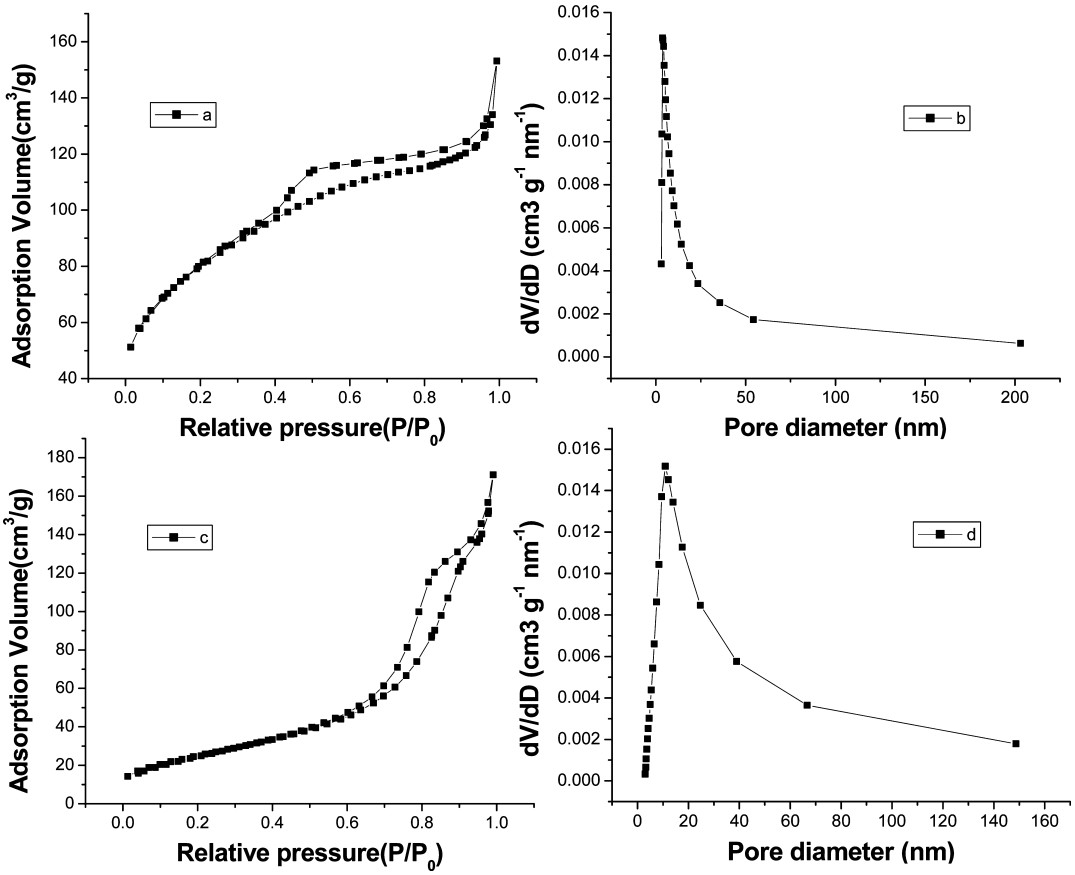

Figure 3. N_2 adsorption−desorption isotherms of (a) Fe_3O_4/graphene/TiO_2 composite precursor and (c) Fe_3O_4/graphene/TiO_2 composites after hydrothermal and calcination treatments. Pore-size distribution of (b) Fe_3O_4/graphene/TiO_2 composite precursor spheres and (d) Fe_3O_4/graphene/TiO_2 composites after hydrothermal and calcination treatments.

Wide-angle X-ray diffraction patterns of the Fe_3O_4/graphene/TiO_2 composite precursor and Fe_3O_4/graphene/TiO_2 composites after hydrothermal and calcination treatments are shown in Figure 2. Before hydrothermal and calcination, TiO_2 nanoparticles are amorphous (Figure 2a). After hydrothermal and calcination treatments, Fe_3O_4/graphene/TiO_2 composites show that the peaks are well matched to that of Fe_3O_4, anatase. Furthermore, an additional diffraction shoulder peak comes from the stacked graphene sheets (Figure 2b).

Additionally, nitrogen sorption at 77 K was used to characterize the porosity of the products. Figure 3a,b shows the N_2 adsorption−desorption isotherm and the pore-size distribution curve for the Fe_3O_4/graphene/TiO_2 composite precursor, and Figure 3c,d displays that of the Fe_3O_4/graphene/TiO_2 composites after solvothermal and calcination treatments. The Fe_3O_4/graphene/TiO_2 composite precursor has a surface area of 208.4 $m^2\ g^{-1}$ and a pore volume of 0.163 $cm^3\ g^{-1}$. The Fe_3O_4/graphene/TiO_2 composites after solvothermal and calcination treatments have a surface area of 95.78 $m^2\ g^{-1}$ and a pore volume of 0.253 $cm^3\ g^{-1}$. As described in our previous work,[39] in comparison to that of amorphous TiO_2 material and commercial TiO_2 material, mesoporous TiO_2 microspheres with the highest surface area has the best enrichment effect, indicating that a larger surface area can lead to a better enrichment result. In this work, the specific surface area of Fe_3O_4/graphene/TiO_2 composites is higher than that of mesoporous TiO_2 in our previous work, and the following experimental results also demonstrate that our new Fe_3O_4/graphene/TiO_2 composites have better enrichment ability.

3.2. Selective Capture of Phosphopeptides Using Fe_3O_4/Graphene/TiO_2 Composites. We first used the Fe_3O_4/graphene/TiO_2 composites to extract phosphopeptides from a solution of peptides resulting from the digestion of β-casein (with a trace of α-casein). β-casein (4×10^{-7} M) digests were directly analyzed by MS, and the MS result shows that only nonphosphorylated peptides were detected (Figure 4a). However, after the enrichment using the novel material, phosphopeptides from β-casein were detected (Figure 4b). In addition, three α-casein phosphopeptides were also detected. Moreover, nonphosphorylated peptides were not detected. These results are shown in Table 1 and indicate that the Fe_3O_4/graphene/TiO_2 composites have a highly selective enrichment efficiency.

Next, we further demonstrated the enrichment specificity of the Fe_3O_4/graphene/TiO_2 composites for phosphopeptides. β-casein (4×10^{-8} M), ovalbumin (4×10^{-8} M), and bovine serum albumin (BSA, 2×10^{-6} M) in a ratio of 1:1:50 were mixed and digested. The obtained peptide solution was enriched and analyzed using the novel material. The analysis results are shown in Figure 5. Almost no nonphosphopetides were detected in Figure 5b, and phosphopetides from the two phosphoprotein digestion solutions were successfully enriched and detected (Table 1). This suggests that Fe_3O_4/graphene/TiO_2 composites have a good enrichment specificity for phosphopeptides.

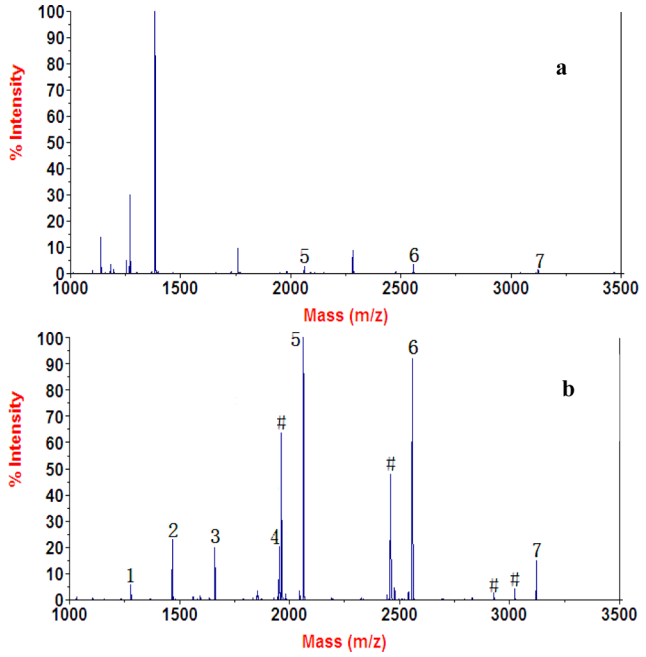

Figure 4. MALDI-TOF mass spectra of the peptides derived from β-casein (a) without enrichment and (b) enriched by Fe_3O_4/graphene/TiO_2 composites. Phosphopeptide ions identified are indicated by numbers. The metastable losses of phosphoric acid are shown by the # symbol.

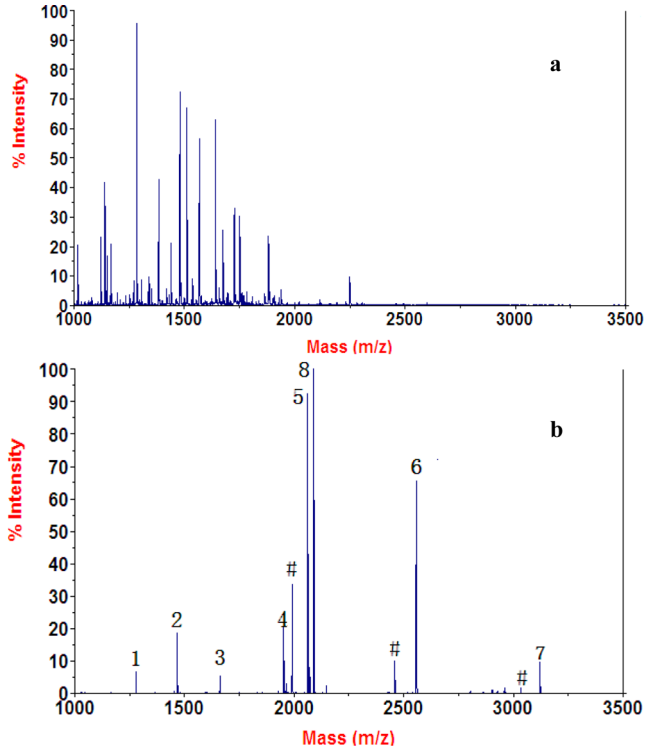

Figure 5. MALDI-TOF mass spectra of peptides derived from a peptide mixture of β-casein, ovalbumin, and BSA at a molar ratio of 1:1:50 (a) without enrichment and (b) enriched using Fe_3O_4/graphene/TiO_2 composites.

To investigate the detection limit of the enrichment ability of the novel materials, we digested β-casein and obtained different concentrations of peptide solutions. The analytical results are shown in Figure S3. It can be seen that after enrichment using the novel material the phosphopeptides from the β-casein digestion (2×10^{-10} M) can still be detected by MS. This shows that the Fe_3O_4/graphene/TiO_2 composites can enrich low-concentration phosphopeptides for use in MS analysis.

We further tested the performance of the Fe_3O_4/graphene/TiO_2 composites for their ability to selectively extract and enrich for phosphopeptides using human liver cancer tissue from a live cancer patient. After searching the database, we obtained the information for the enriched phosphopeptides. Three hundred forty nine phosphorylation sites (253 on serine (72.49%), 82 on threonine (23.49%), and 14 on tyrosine (4.01%)) of 214 phosphopeptides were identified from the cancer tissue. The detailed information for the phosphopeptides is listed in Table S1. To find the probable markers of liver cancer, many studies and control experiments should be performed in the future.

4. CONCLUSIONS

In this work, Fe_3O_4/graphene/TiO_2 composites with a large specific surface area were synthesized, and their phosphopeptide enrichment ability was demonstrated. We first used standard protein digestion to test their enrichment ability for phosphopeptides. Next, we developed Fe_3O_4/graphene/TiO_2 composites for the enrichment and analysis of phosphoproteins from human liver cancer tissue; more than 200 phosphopeptides were found, further proving the effectiveness of this method.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, SEM and EDX pattern of Fe_3O_4/graphene/TiO_2 composites, MALDI-TOF spectra of phosphopeptides enriched from β-casein using Fe_3O_4/graphene/TiO_2 composites, and detailed information for the phosphopeptides

Table 1. Detailed Information for the Observed Phosphopeptides Obtained from Tryptic Digestion of α-Casein S1 and S2, β-Casein, and Ovalbumin[a]

peak no.	theoretical m/z	observed m/z	aa	peptide sequence
1	1278.6	1278.9	β/33−52	FQSEEQQQTEDELQDKIHPF
2	1466.6	1466.7	α-S2/153−164	TVDMSTEVFTK
3	1660.8	1660.9	α-S1/106−119	VPQLEIVPNSAEER
4	1952.0	1952.1	α-S1/104−119	YKVPQLEIVPNSAEER
5	2061.8	2061.8	β/33−48	FQSEEQQQTEDELQDK
6	2556.1	2556.2	β/33−52	FQSEEQQQTEDELQDKIHPF
7	3122.3	3122.3	β/1−25	RELEELNVPGEIVESLSSSEESITR
8	2088.9	2089.0	Ova/340−359	EVVGSAEAGVDAASVSEEFR

[a]The phosphorylation sites are underlined.

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enriched from tryptic digests of liver cancer tissue using Fe_3O_4/graphene/TiO_2 composites. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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