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RESEARCH ARTICLE

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A New Fluorescence Turn-on Assay for Trypsin and Inhibitor Screening Based on Graphene Oxide

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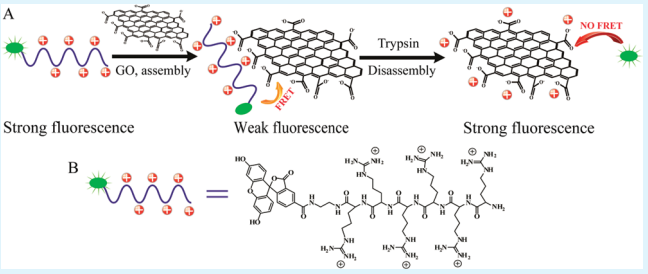
Supporting Information

ABSTRACT: In this paper, we describe a new continuous fluorescence turn-on method for trypsin assay and inhibitor screening in situ. This assay is designed based on the following assumptions: (1) It is expected that the fluorescein-labeled peptide composed of six arginine residues (Arg_6-FAM) with positive charges will interact with the negatively charged edge of water-soluble graphene oxide (GO) because of electrostatic interactions to form a GO/Arg_6-FAM complex. As a result, the fluorescence of fluorescein will be quenched because of the energy transfer from fluorescein to GO. (2) Arg_6-FAM can be hydrolyzed into small fragments in the presence of trypsin, and accordingly, the GO/Arg_6-FAM complex will be dissociated, gradually leading to fluorescence recovery for the solution. In this way, the trypsin activity can be easily assayed with the ensemble of Arg_6-FAM and GO. Additionally, the ensemble can be employed for screening of the inhibitors of trypsin.



KEYWORDS: graphene oxide, fluorescein-labled peptide, trypsin assay, inhibitor screening, fluorescence quenching, fluorescence turn-on

INTRODUCTION

Trypsin, known as the most important digestive enzyme, is produced in the pancreas as the inactive proenzyme trypsinogen.[1] It is a class of protease that plays a key role in controlling the pancreatic exocrine function, and the level of trypsin increased with some types of pancreatic diseases.[2−4] Trypsin is also employed for the degradation of proteins into peptides in proteomics, in particular in mass spectroscopy based proteomics.[5−8] Therefore, new convenient assays for trypsin and its inhibitor screening are highly desired for the development of efficient diagnostic and therapeutic methods toward these pancreatic diseases and application in the proteomics area. Several methods for trypsin assay were reported based on solid-phase cleavage, turbidity, radiometry, and electrochemistry.[9−12] However, they either are time-consuming or require specialized instruments. Fluorometric assay methods were also reported for trypsin based on labeled substrate peptides,[13−18] a water-soluble conjugated polymer,[19−21] natural fluorophore epicocconone,[22,23] and fluorescence polarization.[24−26] We have recently described a label-free continuous fluorescence assay method for trypsin and inhibitor screening based on the aggregation-induced emission (AIE) feature of tetraphenylethene.[27,28]

Graphene, which is a single-atom-thick and two-dimensional carbon nanomaterial, has attracted much attention because of its extraordinary electronic, mechanical, and thermal properties,[29−32] which make it a wide prospect for biological application.[33−36] The oxide form of graphene, graphene oxide (GO), exhibits good water solubility and long-range nanoscale energy-transfer properties.[37,38] By utilization of this property and unique DNA/GO interactions, several biosensors for DNA, proteins, Hg^{2+}, Ag^{+}, and so on have been reported.[39−44] However, so far, few GO-based biosensors have been reported that do not involve DNA. Herein, we report a new fast continuous fluorescence turn-on assay for trypsin and inhibitor screening by making use of the features of GO.

The construction rationale for this fluorometric assay based on GO is schematically illustrated in Scheme 1. GO can be considered as an amphiphile with a largely hydrophobic basal plane and hydrophilic edges. The dispersity and hydrophilicity of GO have been mainly attributed to the ionizable edge COOH groups.[45,46] In a phosphate-buffered saline (PBS; pH = 8.5) buffer solution, the edges of GO will have many negatively charged COO^{-} groups, which is confirmed by the ζ potential measurement for GO in a PBS buffer solution, as depicted in Figure S1 in the Supporting Information. Arg_6-FAM is a fluorescein-labeled peptide composed of six arginine residues with positive charges. The assay for trypsin activity is based on the ensemble of GO and Arg_6-FAM, and the design rationale is explained as follows: (1) It is anticipated that Arg_6-FAM in the solution will interact with GO, mainly because of electrostatic interaction between the COO^{-} groups of GO and the positively charged arginine residues, to form a GO/Arg_6-FAM complex. Accordingly, the fluorescence of the fluorescein unit in

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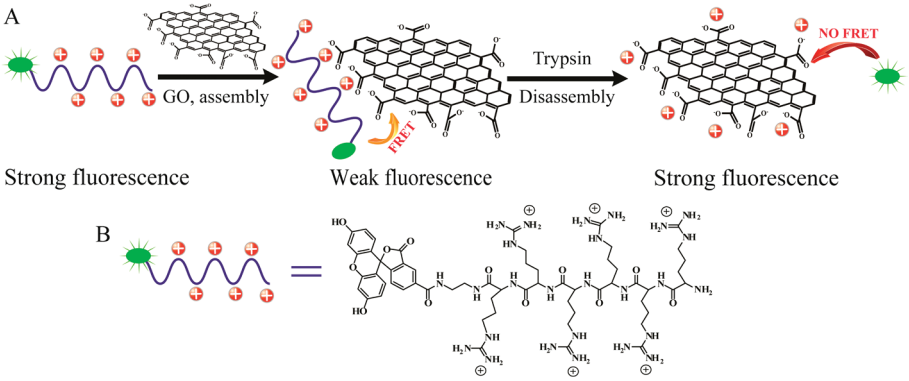
Scheme 1. (A) Illustration of the Design Rationale for the Fluorescence Turn-on Assay for Trypsin with the Ensemble of GO and Arg_6-FAM and (B) Chemical Structure of Arg_6-FAM



Arg_6-FAM will be quenched by GO because of energy transfer from fluorescein to GO.[37,38] (2) Arg_6 can be hydrolyzed into small fragments in the presence of trypsin. Thus, it is expected that the GO/Arg_6-FAM complex will be dissociated when trypsin is added to the solution. As a result, the fluorescence from fluorescein will be restored. Therefore, a new fluorescence turn-on assay for trypsin can be established with the ensemble of GO/Arg_6-FAM. In addition, the hydrolysis of Arg_6-FAM catalyzed by trypsin can be inhibited by trypsin inhibitors. As a consequence, dissociation of the GO/Arg_6-FAM complex will be retarded after a further introduction of the inhibitors, and the fluorescence will be changed accordingly. Therefore, the GO/Arg_6-FAM complex can also be employed for screening of the inhibitors of trypsin.

EXPERIMENTAL SECTION

The Arg_6-FAM peptide was purchased from Invitrogen Trading Co. Ltd. (Shanghai) and purified with high-performance liquid chromatography. The trypsin (1:250) from porcine pancreas was purchased from Biodee Biotechnology Co. Ltd. (Beijing), and the BBI inhibitor [trypsin−chymotrypsin inhibitor from a *Glycine max* (soybean)-lyophilized powder] was purchased from Sigma-Aldrich. All of the chemicals obtained from commercial sources were analytically pure and were used without further purification. Stock solutions of Arg_6-FAM, trypsin, and BBI were prepared with pure water. The pure water used was purified with a Millipore filtration system. A stock solution of GO was prepared with a phosphate-buffered saline (PBS) buffer solution (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM). Sample solutions containing a PBS buffer (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM) for spectral studies were further prepared. The addition of Ca^{2+} is to activate trypsin and increase its stability against autolysis according to a previous report.[47] PBS was prepared by mixing of an aqueous solution of Na_2HPO_4 (179 mg in 250 mL of water) with a certain amount of HCl according to the pH value.

Fluorescence spectra were recorded on a Hitachi F-4500 spectrophotometer with an excitation wavelength at 490 nm. ζ potential measurements were carried out at 25 °C with a Nano ZS (Malvern) equipped with a solid-state He−Ne laser (λ = 633 nm). Atomic force microscopy (AFM) measurements were carried out with a Nanoscope IIIa instrument (Digital Instruments) operating in contact mode.

Synthesis of GO. GO was prepared according to Hummer's method.[52] Characterization of GO by AFM is shown in Figure S2 in the Supporting Information. The thickness of most of GO is about 1.2 nm.

Fluorescence Quenching Experiments by GO. Different amounts of a GO stock solution (0.04 mg/mL) were added into 1.0 mL of a solution of Arg_6-FAM {2.0 μM in a PBS buffer (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM)} to give solutions for which the concentrations of GO varied from 0.00 to 2.20 μg/mL. Each solution was incubated at 37 °C for ca. 2.0 min, followed by recording of the fluorescence spectrum of each solution.

Trypsin Assay with the GO/Arg_6-FAM Ensemble. Different amounts of a trypsin stock solution (1.0 mg/mL) were added into 1.0 mL of a solution of the GO/Arg_6-FAM ensemble {$[Arg_6$-FAM] = 8.5 μM and [GO] = 10 μg/mL in a PBS buffer (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM)} to give solutions for which the concentrations of trypsin were 0.0, 0.1, 0.5, 1.5, 3.0, 6.0, and 10.0 μg/mL. Each solution was incubated at 37 °C, and the fluorescence spectrum was recorded.

Trypsin Inhibition Experiments by BBI Using the GO/Arg_6-FAM Ensemble. Different amounts of BBI were added into 1.0 mL of a solution of the GO/Arg_6-FAM ensemble {[Arg6-FAM] = 8.5 μM and [GO] = 10 μg/mL in a PBS buffer (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM)} to give solutions for which the concentrations of BBI were 0.0, 0.05, 0.2, 0.5, 1.0, and 2.0 μg/mL. To each of the above-mentioned solutions was added 3.0 μL of trypsin (1.0 mg/mL). Each solution was incubated at 37 °C, and the fluorescence spectrum was recorded.

RESULTS AND DISCUSSION

GO was prepared according to a reported procedure,[48] and it can be well dispersed in water. The AFM image (see Figure S2 in the Supporting Information) of as-prepared GO was similar to those reported previously. Characterization of Arg_6-FAM was also provided in the Supporting Information. First, we examined the fluorescence quenching behavior of Arg_6-FAM by GO. Figure 1A shows the gradual fluorescence decrease of Arg_6-FAM after the addition of GO to the solution. For instance, the solution of Arg_6-FAM in the absence of GO shows a strong fluorescence emission (see Figure 1A, curve a). However, after the addition of GO (2.20 μg/mL), up to 99.9% quenching of the fluorescence was observed (see Figure 1A, curve b). Such a fluorescence change can be distinguished by the naked eye, as depicted in the inset of Figure 1A, where the photographs (under UV light irradiation) of two PBS buffer solutions of Arg_6-FAM in the absence and presence of GO are displayed. The corresponding Stern−Volmer plot[49] (F_0/F at 526 nm vs [GO], where F_0 is the initial fluorescence intensity of Arg_6-FAM and F is

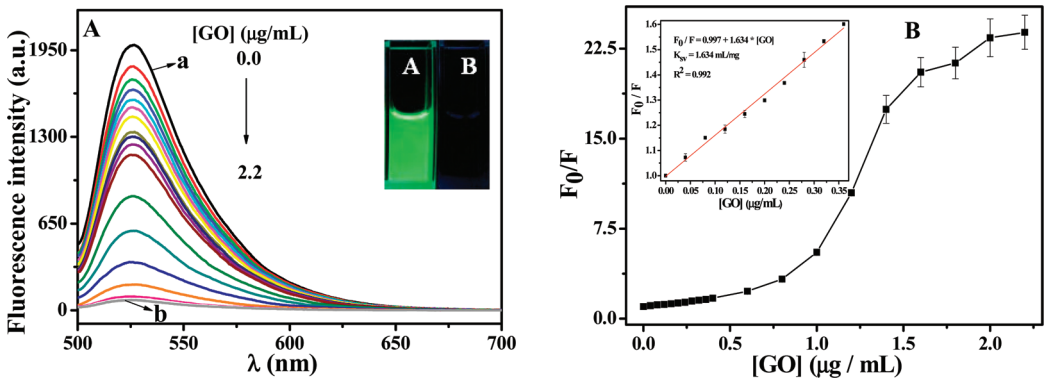


Figure 1. (A) Fluorescence spectra of Arg_6-FAM (2.0 μM) after the addition of different amounts of GO. The inset shows photographs of the solutions of (A) Arg_6-FAM and (B) Arg_6-FAM and GO under UV (365 nm) illumination. (B) Variation of the fluorescence intensity ratios (F_0/F) at 526 nm versus the concentration GO. The inset shows the almost linear plot of the fluorescence intensity ratios (F_0/F) at 526 nm versus the concentration of GO (0.00, 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, 0.28, 0.32, and 0.36 μg/mL) for the solutions in which the concentrations of GO were low. All samples were prepared with a PBS buffer solution (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM), and the excitation wavelength was 490 nm.

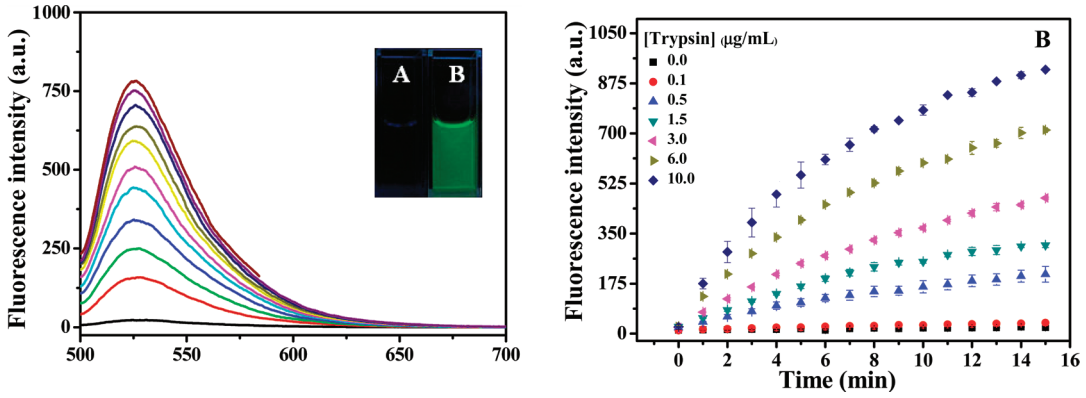


Figure 2. (A) Fluorescence spectra of the ensemble of Arg_6-FAM (8.5 μM) and GO (10 μg/mL) in the presence of trypsin (10 μg/mL) after incubation at 37 °C for different times. The inset shows photographs of the corresponding solutions of Arg_6-FAM and GO without (A) and with (B) trypsin after incubation for 15 min under UV (365 nm) illumination. (B) Plots of the fluorescence intensity at 526 nm versus the reaction time for the ensemble of Arg_6-FAM (8.5 μM) and GO (10 μg/mL) in the presence of different concentrations of trypsin (0.0, 0.1, 0.5, 1.5, 3.0, 6.0, and 10.0 μg/mL). All samples were prepared with a PBS buffer solution (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM), and the excitation wavelength was 490 nm.

the fluorescence intensity of Arg_6-FAM after the addition of GO, is shown in Figure 1B; the corresponding Stern−Volmer constant (K_{sv}) was estimated to be 1.634 $(\mu g/mL)^{-1}$ for the quenching of Arg_6-FAM by GO. Additionally, the fact that the Stern−Volmer plot exhibits an upward-curving feature may indicate the formation of a static complex between Arg_6-FAM and GO.[44] Such fluorescence quenching is probably due to energy transfer from the fluorescein unit in Arg_6-FAM to GO within the GO/Arg_6-FAM complex.[37,38]

Next, experiments were carried out to examine whether the ensemble of GO and Arg_6-FAM can be employed to establish a fluorometric assay for trypsin. As mentioned above, the fluorescence of the GO/Arg_6-FAM complex was very weak (>99% quenching of Arg_6-FAM by GO); moreover, the fluorescence of the complex in a PBS buffer solution did not change with time (see Figure S3 in the Supporting Information), indicating that the complex of GO and Arg_6-FAM was stable. However, Arg_6 can be hydrolyzed in the presence of trypsin, and as a result, the complex of GO and Arg_6-FAM will be dissociated, leading to fluorescence enhancement as discussed above. As shown in Figure 2A, the initial PBS buffer solution of Arg_6-FAM (8.5 μM) and GO (10 μg/mL) showed very weak fluorescence. However, the fluorescence of the ensemble started to increase gradually after the introduction of trypsin into the solution by prolonging the incubation time, as shown in Figure 2A. The fluorescence enhancement was also detected by the naked eye (see the inset of Figure 2A). The fluorescence intensity of the ensemble increased slowly after the solution was incubated at 37 °C for 15 min.

The fluorescence spectra of Arg_6-FAM were also measured in the presence of trypsin under the same incubation conditions. As depicted in Figure S4 in the Supporting Information, the fluorescence intensity change ($F/F_0 - 1$) of Arg_6-FAM, where F_0 and F are the fluorescence intensities at 526 nm before and after the addition of trypsin, respectively, was found to be rather small for Arg_6-FAM. However, a fluorescence intensity change was detected for the ensemble of Arg_6-FAM and GO. These results support the mechanism for the fluorescence enhancement of the ensemble of Arg_6-FAM and GO in the presence of trypsin, as illustrated in Scheme 1.

The fluorescence spectra of the ensemble of Arg_6-FAM and GO were also measured in the presence of different amounts of trypsin. For this purpose, the ensemble of Arg_6-FAM (8.5 μM) and GO (10 μg/mL) was mixed with different amounts of trypsin to give solutions in which the concentrations of trypsin were

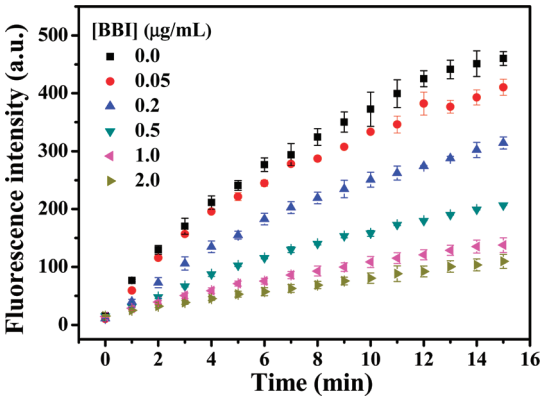


Figure 3. Plots of the fluorescence intensity at 526 nm versus the reaction time for the ensemble of Arg_6-FAM (8.5 μM) and GO (10 μg/mL) containing trypsin (3.0 μg/mL) in the presence of different concentrations of BBI (0.0, 0.05, 0.2, 0.5, 1.0, and 2.0 μg/mL). All samples were prepared with a PBS buffer solution (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM), and the excitation wavelength was 490 nm.

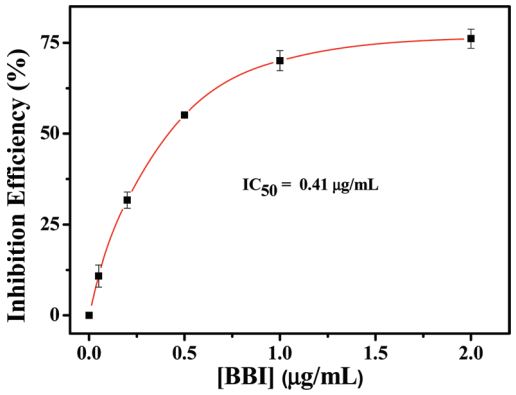


Figure 4. Plot of the inhibition efficiency of BBI toward trypsin versus the concentration of BBI. Measurements were performed with Arg_6-FAM (8.5 μM), GO (10 μg/mL), trypsin (3.0 μg/mL), and different concentrations of BBI (0.0, 0.05, 0.2, 0.5, 1.0, and 2.0 μg/mL) in a PBS buffer (2.0 mM, pH = 8.5, containing $[Ca^{2+}]$ = 10 μM).

0.0, 0.1, 0.5, 1.5, 3.0, 6.0, and 10.0 μg/mL. Each solution was incubated at 37 °C for different times, and then the fluorescence spectrum of each solution was measured. Figure 2B shows plots of the fluorescence intensity at 526 nm versus the reaction time for the ensemble of Arg_6-FAM and GO in the presence of different concentrations of trypsin. The fluorescence of the ensemble remained almost unchanged in the absence of trypsin, but it is obvious that the fluorescence intensity increases after the introduction of trypsin into the ensemble. Moreover, the higher the concentration of trypsin in the solution, the more quickly the fluorescence intensity of the ensemble increased. These results clearly manifest that a continuous fluorescence turn-on assay for trypsin can be established with the ensemble of Arg_6-FAM and GO.

It is understandable that the trypsin-catalyzed hydrolysis of Arg_6-FAM will be retarded in the presence of the corresponding trypsin inhibitors. Therefore, it is expected that the degree of fluorescence enhancement for the ensemble of Arg_6-FAM and GO containing trypsin will become small after the further addition of trypsin inhibitors. BBI, short for the Bowman−Birk inhibitor from soybean,[50] was selected as an example of the inhibitor to illustrate that the ensemble of Arg_6-FAM and GO can also be employed for screening inhibitors of trypsin. Figure 3 displays plots of the fluorescence intensity at 526 nm for the ensemble of Arg_6-FAM (8.5 μM) and GO (10 μg/mL) containing trypsin (3.0 μg/mL) versus the reaction time in the presence of different amounts of BBI (0.0, 0.05, 0.2, 0.5, 1.0, and 2.0 μg/mL). Obviously, the degree of fluorescence enhancement for the ensemble of Arg_6-FAM and GO in the presence of BBI was smaller than that in the absence of BBI. Moreover, the higher the concentration of BBI in the solution, the slower the increase in fluorescence of the ensemble. On the basis of the plot of the inhibition efficiency $[(1 - F/F_0) \times 100\%$, where F_0 and F are the restored fluorescence intensities at 526 nm before and after the addition of inhibitor, respectively],[51] which measures the inhibition ability of the selected inhibitor, versus the concentration of BBI; the corresponding IC_{50} value (the concentration of the inhibitor that leads to 50% inhibition of the enzyme activity) of BBI toward trypsin was estimated to be 0.41 μg/mL (see Figure 4). This IC_{50} value of BBI is lower than that previously reported,[27] which indicates that this new method with the ensemble of Arg_6-FAM and GO is sensitive for screening of the inhibitors of trypsin.

CONCLUSION

In summary, we successfully developed a new continuous fluorescence turn-on method with the ensemble of Arg_6-FAM and GO for a trypsin assay and inhibitor screening. This new assay method is designed by taking advantage of the electrostatic interactions between Arg_6-FAM and GO, the fluorescence quenching ability of GO, and hydrolysis of Arg_6 in the presence of trypsin. The ensemble of Arg_6-FAM and GO can be used to assay trypsin of a concentration as low as 0.1 μg/mL, and thus the sensitivity of this new assay for trypsin is acceptable. Moreover, the ensemble of Arg_6-FAM and GO can be employed for screening of the inhibitors of trypsin with good sensitivity. Both Arg_6-FAM and GO are easily accessible, and analysis can be carried out in aqueous solutions. Given its simplicity, easy operation, sensitivity, and cost-effectiveness, this fluorometric assay is potentially useful for the zhigh-throughput screening of trypsin inhibitors, which may be beneficial for diagnostic methods for pancreatic diseases and anticarcinogenic drug discovery.[52]

ASSOCIATED CONTENT

Supporting Information. Synthesis and characterization of Arg_6-FAM, AFM image for GO, and parts of fluorescence spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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