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ARTICLE TYPE

A new colorimetric strategy for monitoring caspase 3 activity by HRP-mimicking DNAzyme-peptide conjugates

Zhaojuan Zhou, Lu Peng , Xiaoyan Wang, Yu Xiang and Aijun Tong*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

A new method for caspase 3 activity assay has been developed based on HRP-mimicking DNAzyme-peptide conjugates. The mechanism of detection was based on the specific cleavage of DEVD-peptide by active caspase 3 for recognition and the catalytic property of HRP-mimicking DNAzyme for signal amplification. Under optimal conditions, the detection limit of caspase 3 was 0.89 nM. The proposed method was also successfully applied for the detection of caspase 3 in apoptosis cell lysates.

1. Introduction

Proteases, a large family of peptide-bond-cleaving hydrolases, have been extensively studied as they play a vital role in cell-cycle regulation and programmed cell death.[1] Therefore, probing protease activity both *in vitro* and *in vivo* is essential and a number of methods have been developed.[2-11] In this family, caspase 3 is recognized as the most closely related to cellular apoptosis.[12] A tetra-peptide (Asp-Glu-Val-Asp, DEVD) can be specifically recognized and cleaved at the N-terminal of the sequence by caspase 3.[13] By using DEVD-containing peptide coupled with other reactive signal output molecules as sensors, a serial of analytical techniques including fluorescence,[2,14-18] chemiluminescence,[19,20] colorimetry,[21] electrochemistry,[22,23] surface plasmon resonance[24] have been successfully applied for the detection of caspase 3 activity. Among them, the FRET sensor is one of the most studied. For example, a FRET peptide-based oligodeoxyfluoroside probe for measuring caspase 3 activity has been developed.[2] FRET fluorophore-quencher pair was separated by caspase 3 induced cleavage of the linking peptide, thereby producing an enhanced fluorescent signal. However, most FRET-based sensor designs generally require a fluorophore and a quencher labelled at the both ends of DEVD-containing peptides. The labelling not only is expensive but also complicated.[2,15-17] A colorimetric method for the detection of caspase 3 activity has been proposed using an unlabelled DEVD peptide and unmodified gold nanoparticles based on the color changes of gold nanoparticles.[21] This colorimetric assay is label-free, simple and efficient, but the gold nanoparticles are subjective to detection environment such as pH and salt concentrations. An electrochemical detection method has also been proposed by using quantum dots-based signal amplification.[23] However, many of the reported caspase 3 sensors require either expensive reagents (e.g. fluorescent tags), complicated instruments or laborious operation procedures.

On the other hand, horseradish peroxidase (HRP)-mimicking DNAzyme, a single-stranded guanine-rich nucleic acid (PS2.M),[25] exhibits HRP-mimicking activity that catalyzes the oxidation of colorless 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonate disodium salt ($ABTS^{2-}$) by H_2O_2 to produce the colored radical ion ($ABTS^{\cdot-}$).[26] Recently, HRP-mimicking DNAzymes have been used as catalytic labels for the colorimetric detection of microRNAs.[27] Besides, aptamers have been conjugated with HRP-mimicking DNAzymes for amplified detection of a serial target molecules by target-induced activation of the DNAzymes.[28] Furthermore, colorimetric assay for T4 polynucleotide kinase activity was also established based on the HRP-mimicking DNAzymes combined with λ exonuclease cleavage.[29] Although such HRP-mimicking DNAzymes have been utilized for the detection of a number of analytes including metal ions (Pb^{2+}, K^+, Hg^{2+}),[30-35] small molecules (AMP),[28] proteins (lysozyme, thrombin),[28,36] enzymes (telomerase, methyltransferase, hOGG1)[37-39] and nucleic acids,[40,41] to our best knowledge, its application for protease activity assays is still not explored.

Herein, we report a new colorimetric strategy for sensitive detection of caspase 3 activity and its inhibition, through combining the color-generating reaction catalyzed by HRP-mimicking DNAzyme and the specific recognition and cleavage of DEVD peptide by active caspase 3.

2. Experimental

2.1 Materials

The DNAzyme 5'-GGGTAGGGCGGGTTGGGAAAAAA-NH_2-3' was obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). The biotinylated DEVD-peptide (biotin-Gly-Tyr-Tyr-Asp-Gly-Asp-Glu-Val-Asp-Gly-Cys) was synthesized by GL Biochem. Ltd. (molecular weight 1418.49, Shanghai, China). Caspase 3 was purchased from Sigma-Aldrich, Inc. (St. Louis, USA). Streptavidin-coated magnetic beads (mean diameter 1.5 μm) and Amicon centrifugal filters (3K and 10K Da molecular weight cutoff) were purchased from Bangs Laboratories Inc. (Fishers, USA) and Millipore Inc. (Billerica, USA), respectively.

Apoptosis inducers kit and caspase 3 inhibitor (Ac-DEVD-CHO) were purchased from Beyotime Institute of Biotechnology (Haimen, China). Caspase 3 cellular activity assay kit was obtained from Nanjing Kengen Biotechnology Co., Ltd. (Nanjing, China). Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) and BCA protein assay kit were obtained from Thermo Scientific Pierce, Inc. (Rockford, USA). Tris (2-carboxyethyl)phosphine hydrochloride (TCEP) and other chemicals were purchased from Alfa Aesar Co., Ltd. (Tianjin, China). All solutions were prepared using Milli-Q water.

2.2 Characterization

UV-Vis spectra was recorded on a JΛSCO V-550 UV-Vis spectrophotometer. MALDI-TOF MS data was obtained using a Shimadzu AXIMA performance matrix-assisted laser desorption ionization time-of-flight mass spectrometer.

2.3 HRP-mimicking DNAzyme-peptide conjugation

A bifunctional cross linker sulfo-SMCC was used to functionalize biotin-peptide with HRP-mimicking DNAzyme. 2 mg sulfo-SMCC was added to 200 μL of 150 μM DNAzyme in PBS buffer(0.1 M sodium phosphate buffer, pH 7.3, 0.1 M NaCl). After vortexing for 5 minutes, the solution was reacted for 1 h at room temperature. Then excess insoluble sulfo–SMCC was removed by centrifugation. The solution was then purified by Amicon 3K using PBS buffer. On the other hand, 40 μL of 1 mM biotin-peptide was first incubated with three equivalent of TCEP. Then the purified biotin-peptide was mixed with the above solution of DNAzyme. The resulting solution was kept at room temperature for 48 h. After that the solution was purified by Amicon 3K 8 times using PBS buffer.

2.4 Procedures for caspase 3 activity detection

Sensor preparation. A portion of 500 μL 1mg mL^{-1} streptavidin coated magnetic beads (MBs) were buffer exchanged to PBS buffer (0.1 M sodium phosphate buffer, pH 7.3, 0.1 M NaCl, 0.05% Tween-20) twice using a magnetic rack. Then 500 μL of 15 μM DNAzyme-peptide conjugates were added to the dry MBs. The mixture was mixed on a shaker for 1 h at room temperature. After washing the MBs residue by 3 times using PBS buffer containing 2 mg mL^{-1} BSA to block nonspecific binding sites by BSA, each portion of 50 μL 1mg mL^{-1} MBs in PBS buffer was separated.

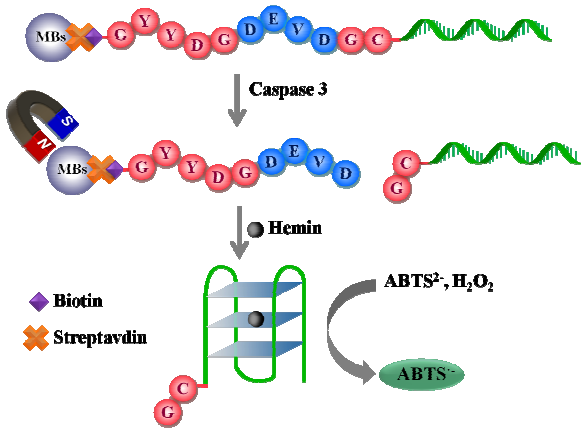
Caspase 3 detection. Different concentrations of target caspase 3 in 50 μL reaction buffer (50 mM HEPES, 100 mM NaCl, 20 mM KCl, 0.03% Triotin X-100, pH 7.4) was added to each portion of the above 50 μL MBs residues and incubated at 37 °C for 2 h. After reaction, the solution was collected by a magnetic rack. 1 μL of 100 μM hemin was added to the solution and reacted for 30 min to insure hemin bind to DNAzyme. Then 25 μL of 8 mM ABTS and 25 μL of 8 mM H_2O_2 were added. The absorption spectra were recorded with a JΛSCO V-550 UV-Vis spectrophotometer at 5 min.

2.5 Preparation of Hela cell lysates

Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and maintained at 37 °C in an atmosphere of 5% CO_2. 4 mL of Hela cells (1.3×10^6 cells mL^{-1}) was incubated with 4 μL apoptosis inducer A for 0 h, 4 h, 8 h, 12 h, respectively. Normal Hela cells without apoptosis inducer were as the control studies. Then cells were collected using a scraper and washed by PBS buffer twice. 200 μL lysis buffer was added into the cell residues and placed on ice for 30 min. After centrifugation, the supernatant was collected as Hela cell lysates. BCA protein assay kit was used to determine total protein of each lysate. 50 μL cell lysates contained 150 μg protein was used for caspase 3 detection.

3. Results and discussions

3.1 Principle of caspase 3 activity detection

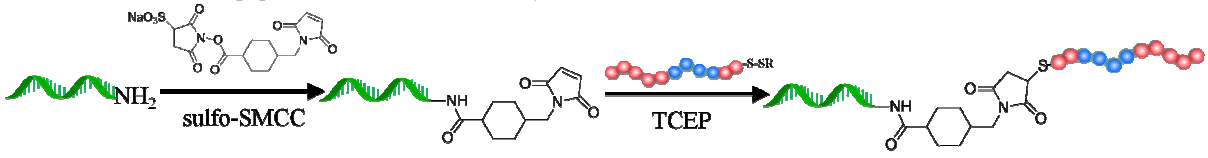


Scheme 1. Schematic illustration of the colorimetric assay for caspase 3.

As shown in Scheme 1, a biotin-peptide (biotin-Gly-Tyr-Tyr-Asp-Gly-Asp-Glu-Val-Asp-Gly-Cys) was designed and four amino acids (in blue) of the peptide were Asp-Glu-Val-Asp (DEVD), which could be recognized and specifically cleaved by active caspase 3. This DEVD-containing peptide was conjugated with an HRP-mimicking DNAzyme. Subsequently, the DNAzyme-peptide conjugates were immobilized onto magnetic beads (MBs) via streptavidin-biotin binding. MBs were used to capture DNAzyme-peptide conjugates and remove excess DNAzyme-peptide conjugates by three times washing through magnetic separation. In the presence of caspase 3, the DNAzyme-peptide conjugates were cleaved at the DEVD site and released the DNAzyme part from MBs. Then the solution was separated using a magnetic rack, the released DNAzyme could then assemble into a G-quadruplex structure with hemin and catalyze the oxidation of $ABTS^{2-}$ to generate a green product $ABTS^{\cdot-}$ for colorimetric signal readout at 420 nm.

To conjugate the DNAzyme with the biotin-peptide, the DNA strand modified with an amine group at its 3' terminus (5'-GGGTAGGGCGGGTTGGGAAAAAA-NH_2-3') was used. This NH_2-DNA was reacted with a bifunctional cross linker sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-

carboxylate (sulfo-SMCC), which contained an amine-reactive N-hydroxysuccinimide and a sulfhydryl-reactive maleimide (shown in Scheme 2). The resulting maleimide-terminated DNAzyme was subsequently conjugated with the thiol in a cysteine residue of the biotin-peptide (in excess amount) to yield DNAzyme-peptide conjugates. Unreacted biotin-peptide was removed by a centrifuge filter of a 3 kDa molecular weight cut-off.



Scheme 2. The conjugation of HRP-mimicking DNAzyme and DEVD-peptide.

3.2 Characterization of HRP-mimicking DNAzyme-peptide conjugates

To verify the desired conjugation product, 10% denative polyacrylamide gel electrophoresis (PAGE) was carried out in 1×TBE buffer (pH 8.0). As illustrated in Fig. 1, three lanes in the PAGE image were loaded from left to right with 60 μM biotin-peptide, 30 μM DNAzyme, and 30 μM conjugation product containing DNAzyme-peptide conjugates, respectively. It was observed from the gel that the band of DNAzyme-peptide conjugates (9.1 kDa) migrated slower than that of DNAzyme (7.5 kDa) or peptide (1.4 kDa) because of its larger molecular weight. The light band in lane 3 had an identical gel mobility as the band in lane 2, indicating there was a little amount of unreacted DNAzyme in the conjugation product. The unreacted DNAzyme did not affect the detection, because it contained no biotin modification and would be washed away during the immobilization of the conjugates onto MBs. The conjugation product was characterized by MALDI-TOF MS (Fig. S1). The m/z of the product was found 9130.4, corresponding well with the calculated value of 9129.6 for the DNAzyme-peptide conjugate.

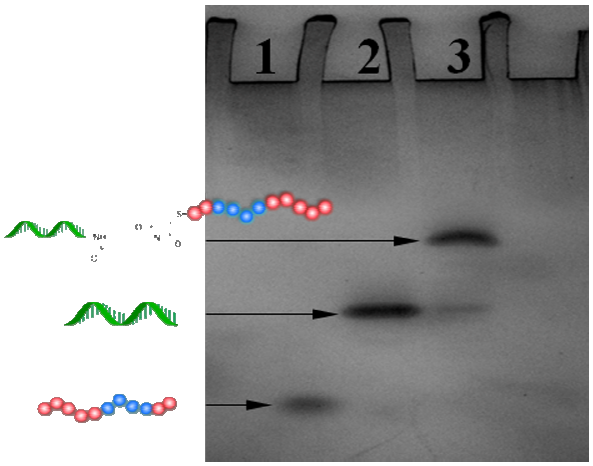
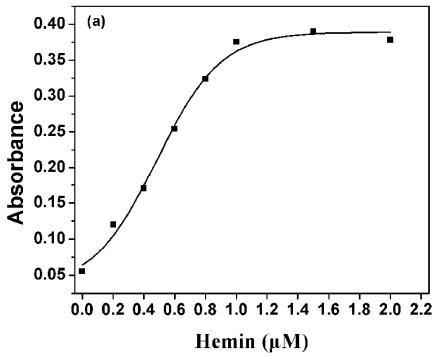


Fig. 1. Denative polyacrylamide gel electrophoresis (10%) image for biotin-peptide (1), DNAzyme (2), and conjugation product (3). The gel was stained by Stains All to visualize both peptide and DNA bands.

The as-prepared DNAzyme-peptide conjugates were immobilized onto MBs via streptavidin-biotin interaction and unbound conjugates were removed via magnetic separation. The amount of conjugates loading on the MBs was determined by the decrease of the DNAzyme part's characteristic absorbance at 260 nm in the supernatant before and after immobilization (Fig. S2). Around 5.3 pmol conjugate was loaded onto 1 mg MBs based on the calculation.

3.3 Optimization of the analytical procedure

Next, the MBs containing the conjugates were used for caspase 3 detection (Scheme 1). The optimal conditions for the detection were first investigated to achieve high analytical performance. Hemin plays an important role in the catalytic activities of HRP-mimicking DNAzymes. Hence, the concentration of hemin was studied first (shown in Fig. 2a). The absorbance at 420 nm as signal readout reached a plateau when 1 μM hemin was added to the test solution containing 1 mg mL^{-1} MBs and 50 nM caspase 3. The effect of reaction time for caspase 3 to cleave the conjugates and release the DNAzyme part from MBs was also tested (shown in Fig. 2b), and 2 h was found sufficient for maximum signal output. Therefore, 1 μM hemin and 2 h reaction time were used for subsequent detections. Furthermore, the concentrations of ABTS and H_2O_2 were also optimized (Fig. S3). The more H_2O_2 may cause oxidative damage of the HRP-mimicking DNAzyme,[25] and excess ABTS will increase the absorbance background. Thus, 2 mM H_2O_2 and 2 mM ABTS were applied in this work.



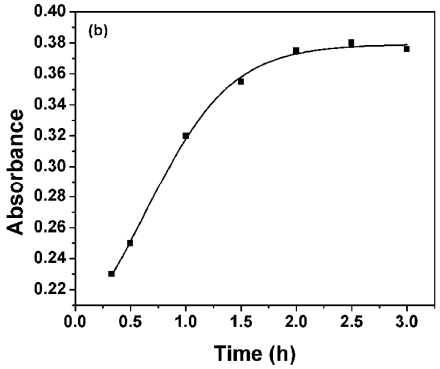


Fig. 2. Condition optimization. (a) effect of hemin concentration, (b) effect of the reaction time between caspase 3 and MBs.

3.4 The sensitivity of this method for caspase 3 activity assay

Under the above optimal condition, the performance of this colorimetric method for caspase 3 detection is shown in Fig. 3. The absorbance at 420 nm increased gradually for samples containing increasing concentrations of caspase 3 from 0 to 50 nM. A detection limit of 0.89 nM (defined by $3\sigma_b$/slope, σ_b is the standard deviation of the blank samples) was achieved under the condition (Fig. 4). This detection limit is comparable or better than some other published methods for caspase 3 activity detection (shown in table 1)[16,18,21,42].

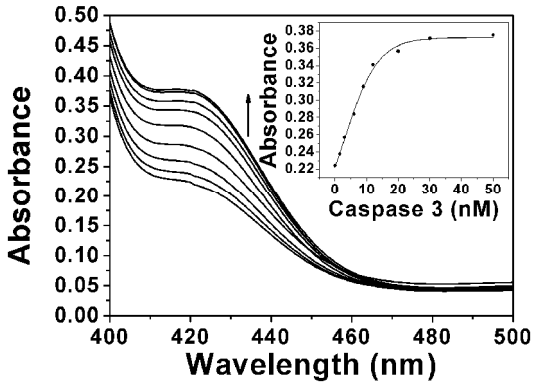


Fig. 3. Absorption spectra responses of the DNAzyme-peptide conjugates to various concentrations of caspase 3, concentration of caspase 3 changes from 0 nM to 50 nM as indicated by the arrow. Inset: Absorbance at 420 nm versus caspase 3 concentration.

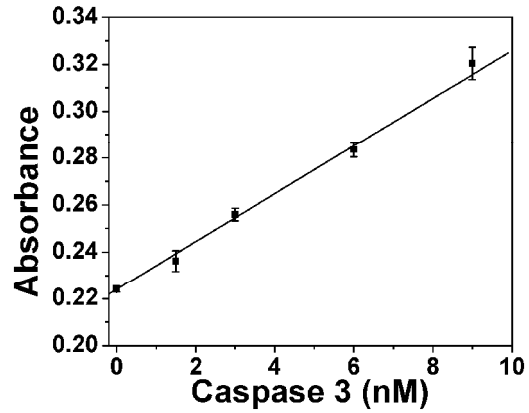


Fig. 4. Calibration curve of caspase 3 detection.

Table 1 Comparison of the detection limit for caspase 3 activity assay

Method	Limit of detection	Reference
Fluorescence,	0.2 ng μL^{-1} (11.1 nM)	[42]
Fluorescence	3.2 nM	[16]
Colorimetry	0.01 $\mu g\ mL^{-1}$ (0.55 nM)	[21]
Fluorescence	0.4 nM	[18]
Colorimetry	0.89 nM	This work

3.5 Inhibition study and Selectivity

Furthermore, to confirm the colorimetric response was indeed due to the catalytic activity of caspase 3, a caspase 3 inhibitor Ac-DEVD-CHO was used to incubate with a sample containing 50 nM caspase 3 for 30 min prior to the tests. As expected, the signal response of the sample containing the inhibitor was much lower than that in the absence of the inhibitor (Fig. 5), indicating the essential role of active caspase 3 on the absorbance enhancement at 420 nm. To evaluate the specificity of the method to caspase 3 over other proteases, thrombin and trypsin were also added to the test solutions instead of caspase 3. The result in Fig. 6 shows that only caspase 3 could induce a significant absorption change, suggesting the high selectivity of the method for caspase 3.

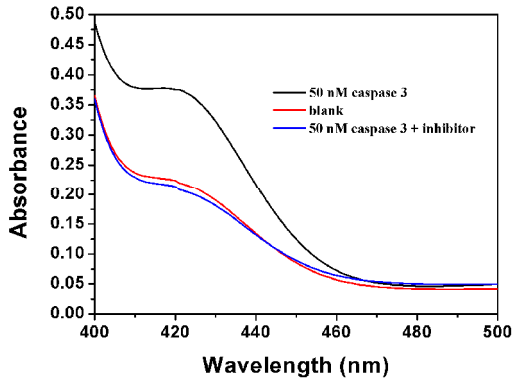


Fig. 5. Caspase 3 activity inhibition by Ac-DEVD-CHO. The blank sample is the buffer solution without target caspase 3.

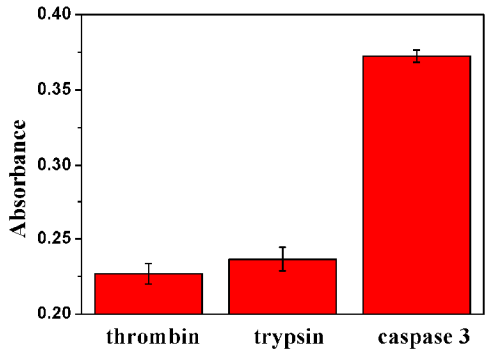


Fig. 6. Absorbance at 420 nm for the method in the presence of different proteases. The concentration of each protease is 50 nM.

3.6 Application in apoptosis induced Hela cell lysates

Finally, owing to the important roles of caspase 3 played in cellular apoptosis, we investigated the application of the method for caspase 3 detection in complex cellular proteomes. Both normal and apoptotic Hela cell lysates were collected and directly treated with 50 µL 1 mg mL^{-1} MBs loaded with DNAzyme-

peptide conjugates. A commercial available caspase 3 cellular activity assay kit was also used and confirmed the presence of active caspase 3 in apoptosis induced Hela cell lysates (Fig. S4). As shown in Fig. 7, the absorbance at 420 nm increased for the cell lysate incubated with the apoptosis inducer for longer time, suggesting more active caspase 3 was generated upon time after inducing. In contrast, control experiments with normal Hela cells in the absence of inducer did not show obviously absorbance enhancement upon incubation. These results indicated that the method could be used for the detection of caspase 3 activity during cell apoptosis.

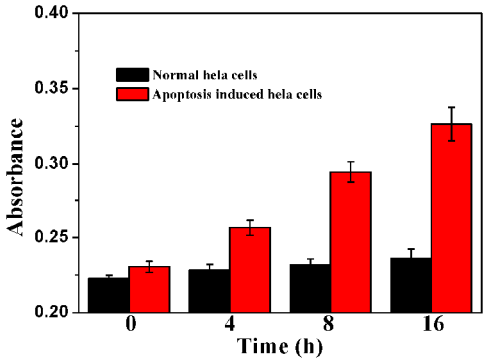


Fig. 7. Detection of caspase 3 in Hela cell lysates with or without apoptosis inducer. Hela cells were incubated with apoptosis inducer for 0 h, 4 h, 8 h, 12 h, respectively.

4. Conclusions

In summary, we have developed a DNAzyme-based method for the colorimetric detection of active caspase 3 with high sensitivity and selectivity. This colorimetric strategy combines the HRP-mimicking DNAzyme-based signal amplification and the specificity of caspase 3 in recognizing and cleaving peptide substrates. The method can be used for the detection of both caspase 3 activity and its inhibitors, and is also successfully applied in caspase 3 activity assays for cell lysate samples to detect active caspase 3 during cell apoptosis. Using suitable peptide sequences instead of DEVD, other proteases can also be tested via the approach in this method.

Acknowledgements

We thank for the financial support from the National Natural Science Foundation of China with the Grant 21175079 and 21375074.

Notes and references

Department of Chemistry, Beijing Key Laboratory for Analytical Methods and Instrumentation, Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Tsinghua University, Beijing 100084, China.
Fax: +86-10-62782485; Tel: +86-10-62787682; E-mail: tongaj@mail.tsinghua.edu.cn
†Electronic Supplementary Information (ESI) available: Experimental details. See DOI: 10.1039/b000000x/

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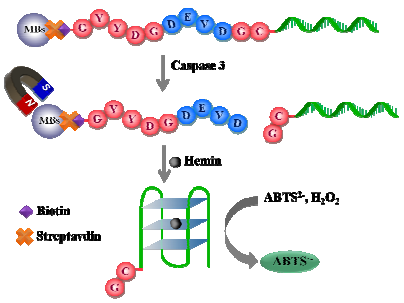

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