Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

EPR based *in situ* enzymatic activity detection of endogenous caspase-3 in apoptosis cell lysates

Aokun Liu[a‡], Jian Kuang[b‡], Yemian Zhou[a], An Xu[a], Changlin Tian[a,b*] and Lu Yu[a*]

[a] High Magnetic Field Laboratory, Chinese Academy of Sciences, Hefei, Anhui 230031, China

[b] The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, Center for Bioanalytical Chemistry, Hefei National Laboratory of Physical Science at Microscale, University of Science and Technology of China, Hefei, Anhui 230026, China

‡ These authors contributed equally to this work.

* Corresponding authors: cltian@ustc.edu.cn(C.L.T.) and luyu@hmfl.ac.cn(L.Y.).

Experimental Procedures

Materials and reagents

Unless otherwise stated, all reagents and solvents used for synthesis were purchased from commercial sources and used without further purification. Streptavidin-modified magnetic beads (MBs) (2.8 μm, 10 mg mL-1) were purchased from Beaverbio Co., Ltd. (Suzhou, China). MBs were washed thoroughly using HEPES buffer (pH 7.5) before conjugation with peptide. The nitroxide spin label, MTSL (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrr-oline-3-methyl methanethiosulfonate), was purchased from Toronto Research Chemicals, Ontario, Canada.

Synthesis and biotinylation of the hexapeptide

The hexapeptide(Ala_{β}-Asp-Glu-Val-Asp-Cys) was synthesized by KS-V Peptide Biological Technology Co., Ltd (Hefei, China) by standard solid-phase peptide synthesis (SPPS) procedure as previously described.[1] In brief, 2-CTC resin was swelled in DMF/DCM 1:1 for 30 min. Fmoc-Cys(Trt)-OH (4 eq, according to the substitution rate of the resin) and DIEA (8 eq) dissolved in DMF and reacted with the resin overnight to load Cys residue onto the resin. Fmoc de-protection was carried out using 20 v%/v% piperidine in DMF twice (5 min + 10 min). Fmoc-Asp(OtBu)-OH, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH were coupled onto the resin followed by Fmoc de-protection successively, coupling condition was Fmoc-AA (4 eq), HCTU (3.8 eq) and DIEA (8 eq) in DMF for 45min. Biotin was introduced to the N-terminus of the peptide under the same coupling conditions as a building block.

The resin was washed thoroughly by DCM and dried in vacuum. TFA cleavage cocktail was poured into the reaction vessel and reacted for 3h. TFA cocktail was filtered into a 50 mL EP tube and TFA was removed by blowing N_2 gas. Cold ether was poured into the tube to precipitate the crude peptide. The Crude peptide was purified by reversed-phase HPLC (eluent: H_2O/acetonitrile containing 0.1% TFA) and characterized by ESI-MS.

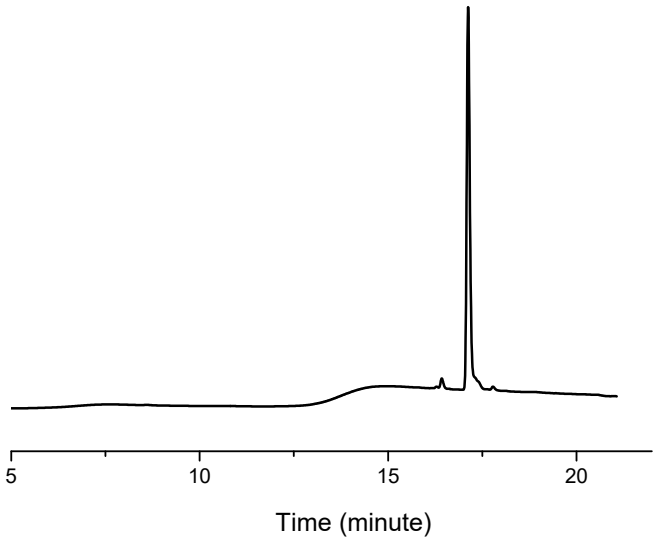

Figure S1. HPLC of peptide Biotin-Ala(beta)-Asp-Glu-Val-Asp-Cys.

Eluent: 0.1% TFA aq. (0 min) → 99% acetonitrile / 1 % water containing 0.1% TFA (30min).

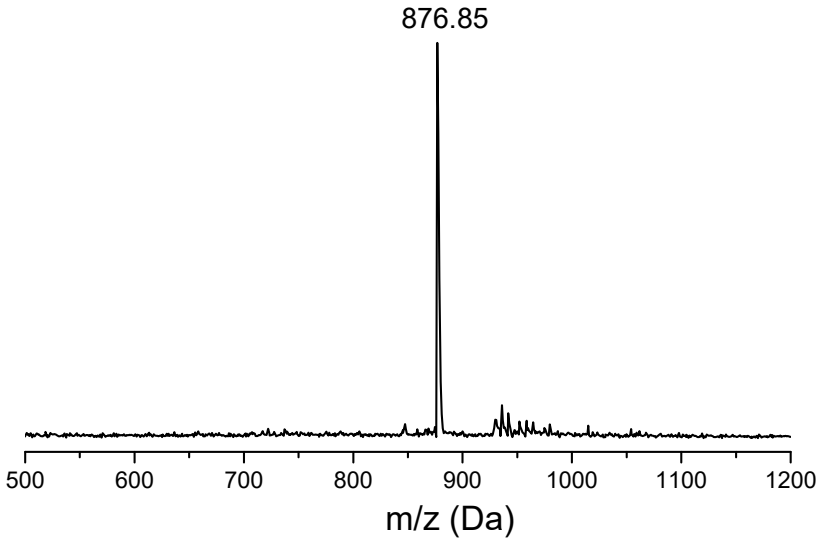

Figure S2. Mass spectroscopy of peptide Biotin-Ala(beta)-Asp-Glu-Val-Asp-Cys.

Spin labeling of the probe

The biotinylated peptide was firstly conjugated to streptavidin-coated magnetic beads by incubation at 4 ℃ for 6 hours in HEPES buffer (pH 7.5). Nitroxide MTSL was then introduced into the thiol groups of the c-terminal cysteine of the peptide with a spin label/peptide molar ratio of 10:1 at 4 ℃, overnight. The excess unbound spin label was removed by washing the MBs with HEPES buffer (pH 7.5) for at least 10 times on a magnetic rack. The successful removal of unbound nitroxide was monitored by measuring the EPR signal of the elution until no EPR signal was detectable.

The “effective probe concentration”, as the number of spins for 1mg of MBs, was calculated by comparison of the EPR signal intensity of the cleaved probe with a series of standard reference samples (spin probe with known concentrations). Briefly, the successfully prepared probe was re-suspended with HEPES buffer and an aliquot of 0.2 mg probe (refer to the weight of MBs) was taken to react with excess caspase-3 (for example, 30 μL of caspase-3 with a concentration of 50 μM) at 37 degrees. The reaction was monitored by EPR and was terminated until the EPR signal of the supernatant doesn’t change anymore, indicating that all probes have been cleaved by the protease. Incubation time of both 30 minutes and 60 minutes was tested in the experiment and the result showed that 30 minutes is long enough for a complete cleavage. Then a series of standard reference samples (MTSL solutions with concentrations at 100 nM, 250 nM, 500 nM, 1 μM, 5 μM, 25 μM, 50 μM) was prepared and their corresponding EPR spectrum was collected. The spectral intensities were calculated by double integration of the EPR spectrum and plotted against the concentrations of MTSL solutions. A linear standard curve (R^2=0.999) between the spectral intensity and spin concentrations was obtained. Then EPR spectra of the supernatant from the enzyme-cleaved probe was recorded using exactly the same parameters as the MTSL solution samples and the spectra intensity was derived by double integration too. The spectra intensity was then plugged into the above-derived linear equation and the spin concentration of the supernatant was then obtained. In this way, the spin concentration of the probe could be obtained for a specific volume of MBs. We repeated the procedure with different aliquots of the prepared probe and the “effective probe concentration” was calculated to be 0.356 nmol for 1 mg of MBs, with standard derivation less than 1% (Figure S3).

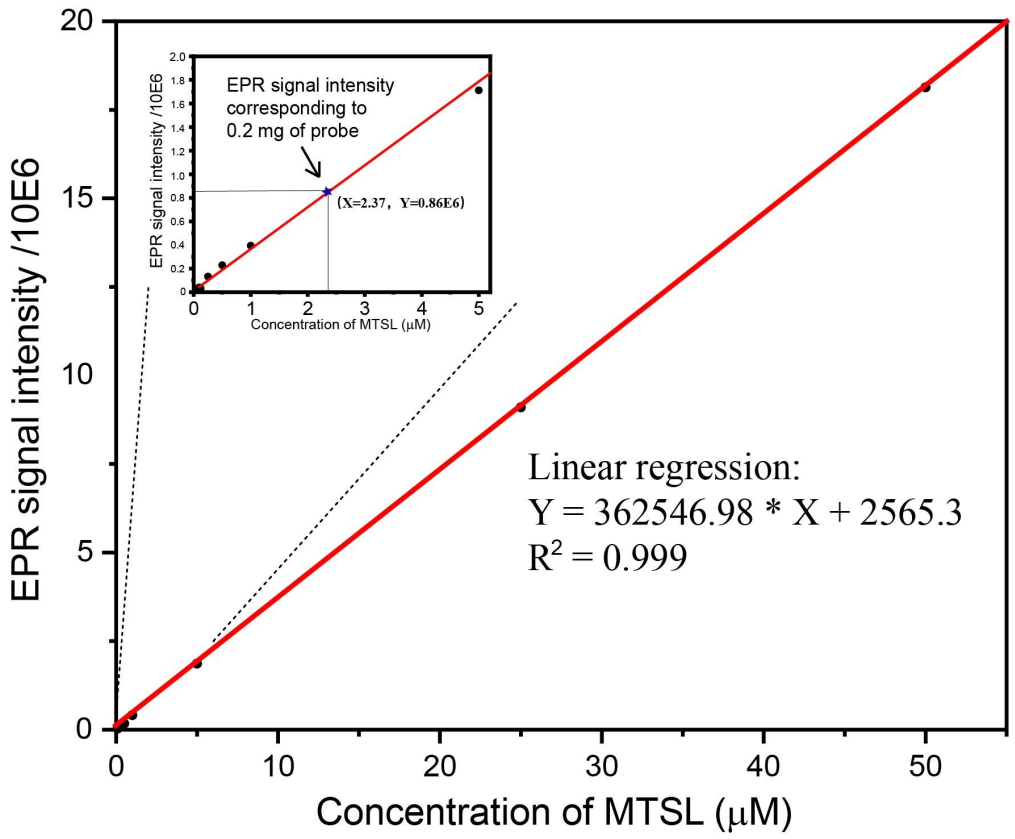

Figure S3. Calculation of the concentration of prepared probe. A linear standard curve was obtained between the spectral intensity and spin concentrations for MTSL solutions with concentrations ranging from 100 nM to 200 μM. EPR spectrum of prepared probe (0.2 mg) incubated with excess caspase-3 (30 μL, 50 μM) for a complete digestion. The EPR spectral intensity of the supernatant of the probe after caspase-3 digestion was plugged into the linear equation and the spin concentration for 0.2 mg of probe was derived (inset). The data points corresponding to 100 μM and 200 μM were omitted for better illustration effect, but were included in the linear regression fitting.

Expression and purification of Caspase-3

Caspase-3 was expressed and purified according to published protocols.[2,3] Briefly, oligonucleotide encoding full-length human caspase-3 protein were synthesized by GENEWIZ (Suzhou, China) and was introduced into the plasmid expression vector pET28a (Novagen). The plasmids were transformed into BL21(DE3) *E. coli.* strain. Single colonies were used to inoculate a 5 ml 2 YT (0.5 g NaCl, 1% yeast extract, and 1.6% tryptone) pre-culture, containing the appropriate antibiotic and was then grown at 37 ℃ overnight (225 rpm). This culture was used to inoculate 500 ml of selective 2 YT medium in 2-liter baffled flasks and grown at 37 ℃. When the culture reached an absorbance of 0.6–0.8 the temperature was lowered to 30 ℃ and expression was induced by the addition of 0.2 mM IPTG. Cells were harvested after 3 hours of incubation and then washed in a buffer containing 100 mM Tris–HCl, pH 8 and 100 mM NaCl and subsequently resuspended in 50 ml lysis buffer (100 mM Tris–HCl, pH 8, 100 mM NaCl, 20 mM imidazole, and 10 units/ml DNase I). Cells were broken using probe sonication and purified using Ni-NTA affinity chromatography (Qia-gen, USA). Nonspecifically bound protein was washed out with 100–200 column volumes wash buffer (50 mM Tris–HCl, pH 8, and 150 mM NaCl, and 20 mM imidazole). The protein was eluted in 0.5 ml fractions with 6 ml elution buffer (50 mM Tris–HCl, pH 8, and 150 mM NaCl, and 250 mM imidazole). Fractions containing pure, active caspase were pooled. The concentration of the purified protein was determined by OD_{280}.

EPR measurement

EPR spectroscopy was performed on a Bruker A300 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) at X-band (9.5 GHz) equipped with a high-sensitivity cavity (ER 4119HS, Bruker Biospin GmbH, Rheinstetten, Germany). Spectra were recorded at a microwave power of 2 mW over a scan width of 65 Gauss with field modulation of 1 Gauss and modulation frequency of 100 kHz through all measurements. EPR spectra were collected at temperature of 37 °C, which was kept using a Bruker variable temperature control unit.

All the enzymatic incubation was carried at 37 ℃. For each sample, the supernatant was collected for EPR measurement after magnetic separation using a magnetic rack. The samples were placed in a glass capillary tube with a fixed volume of 10 μL and a typical EPR spectrum of the released nitroxide is shown in Figure S4, left panel. For the control experiment, the probe was incubated with HEPES buffer, instead of caspase-3, using the same incubation period. EPR spectrum was collected under exactly the same conditions (spectrometer parameters, temperature, etc.) and no peaks were observed (Figure S4, right panel).

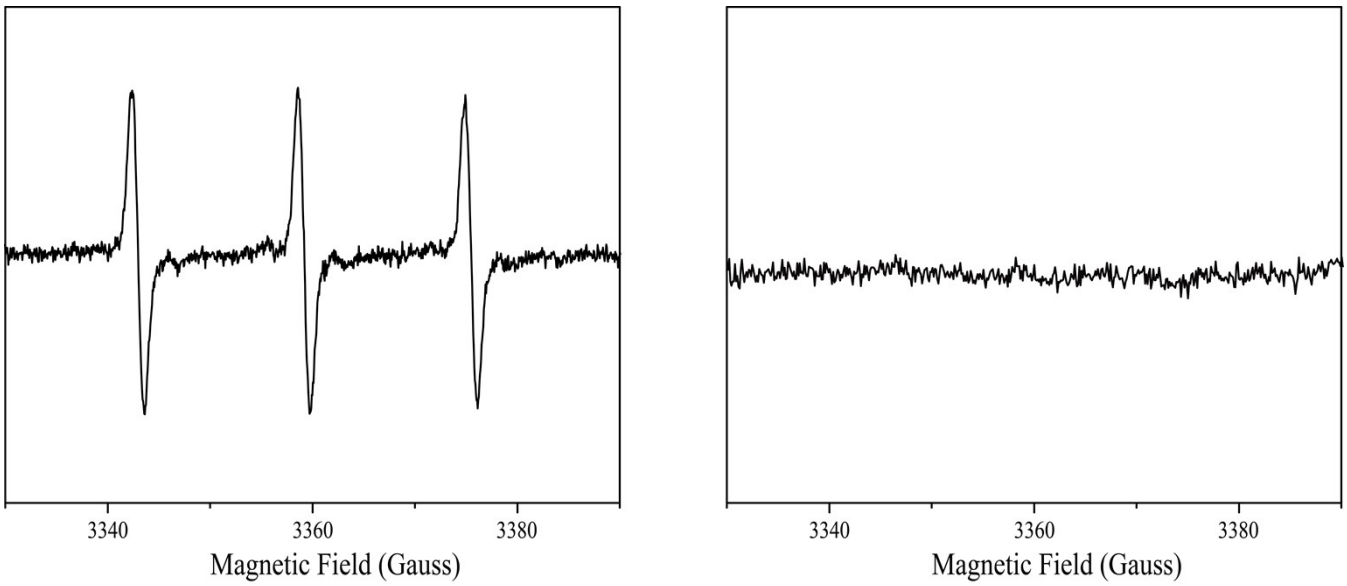

Figure S4. EPR spectra of the supernatant from probe incubated with caspase-3 (left panel) and HEPES buffer (right panel).

For EPR spectrum of the samples from different enzymatic incubation periods (Figure 2a), the enzymatic reactions were quenched at the corresponding time by boiling water, which only deactivate caspase-3 without affecting the linewidth and intensity of EPR spectrum of the released nitroxide, as shown in Figure S5.

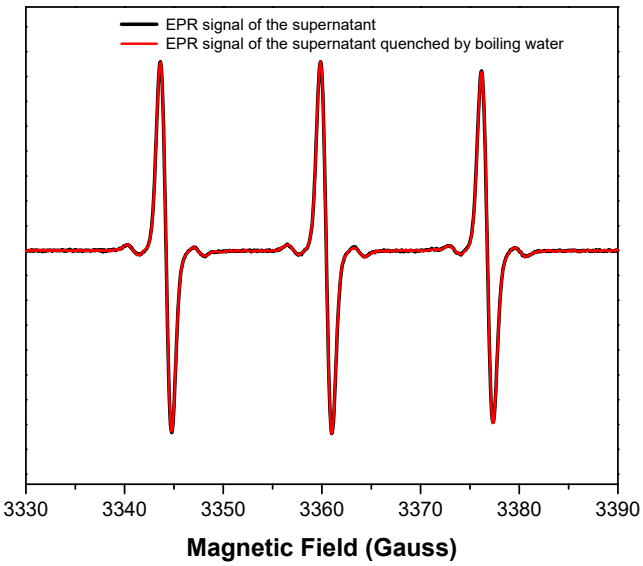

Figure S5. Comparison of EPR spectrum of the released nitroxide before (black trace) and after(red trace) treatment of boiling water(>95 ˚C) for 60 seconds.

Calculation of Michaelis constants k_M and the maximum rate of reaction V_{max}

The Michaelis constants k_M and the maximum rate of reaction V_{max} were estimated by fitting with Eq. (1) and Eq. (2)[4]:

$$V_0 = \frac{V_{\max}[S]}{k_M + [S]} \qquad \text{Eq. (1)}$$

$$V_{\max} = K_{cat}[E_0] \qquad \text{Eq. (2)}$$

Where V_{max} means the maximal rate of the enzyme catalysis reaction at a given concentration of enzyme. k_M, the Michaelis constant, is the concentration of the substrate required to reach half of V_{max}, and has units of M. [S] is the concentration of substrate. V_0 is the initial rate of the reaction while [E_0] means the concentration of the enzyme at the beginning of the reaction. K_{cat} is the first-order rate constant for the enzyme-catalyzed reaction at saturating concentrations of substrate.

Assays were carried out with caspase-3 at a fixed concentration (8.5 μM) and probe at different concentrations(1 μM, 2 μM, 4 μM, 6 μM, 8 μM and 12 μM). The EPR signal intensity was plotted versus the reaction time and initial velocities V_0 were obtained from a linear least-squares fit of the plot in the linear region (the first 10 minutes). The calculated velocities were then plotted versus the concentrations of the probes and the curve was fitted with the Michaelis-Menten equation to yield the Michaelis constants K_M and the maximum rate of reaction V_{max}.

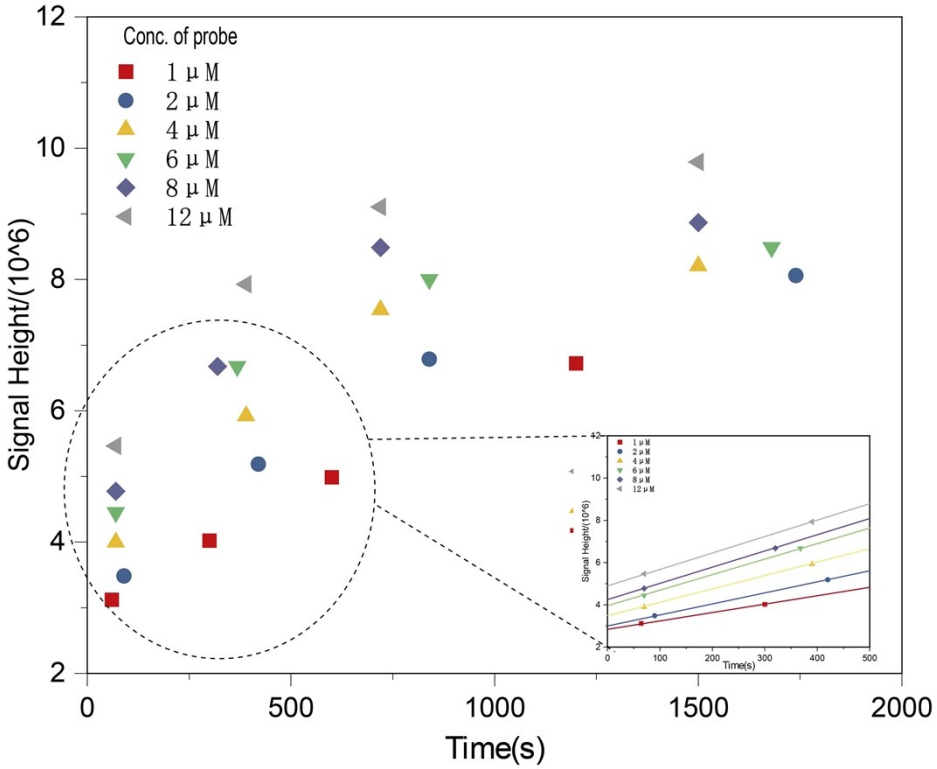

Figure S6. The change in signal intensity of EPR spectrum (calculated as peak-to-peak amplitude of the central peak of EPR spectrum) with different initial concentrations of probe were plotted versus time. Linear least-squares fits of the plot were carried out in the linear region to yield the initial velocities.

Determination of LOD

For the EPR assay reported here, the LOD (Limit of Detection) depends not only on the spectrometer or the EPR method, but also on the observation time (in this case, the incubation time allowed for reaction before EPR measurement). Here, we focused on detection within time scales less than 2 hours. The analysis is based on the facts that the initial apparent rate of the enzymatic reaction (represented by the increase of EPR signal intensity) is proportional to the enzyme concentration. The LOD values for the detection of caspase-3 were estimated according to previously published procedures.[5,6] In brief, the LOD and LOQ (Limit of Quantitation) of the released nitroxide were first determined by measuring the EPR spectrum of blank sample (in this case, HEPES buffer containing caspase-3) and released nitroxide (in this case, MTSL-labelled cysteine, designated as C-MTSL). The LOD of released nitroxide was calculated using the equation LOD = 3δ/m where δ is the standard deviation of the blank sample and m is calculated from linear fitting of the slope shown in Figure S8.

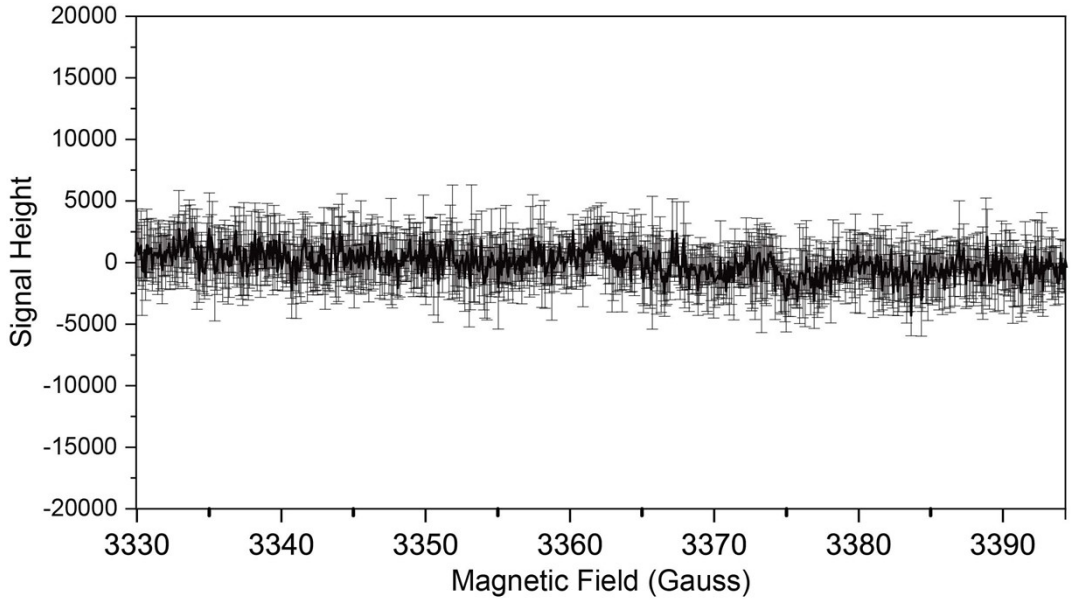

Figure S7. Buffer containing Caspase-3 as blank sample (error bars: standard deviation, n=5).

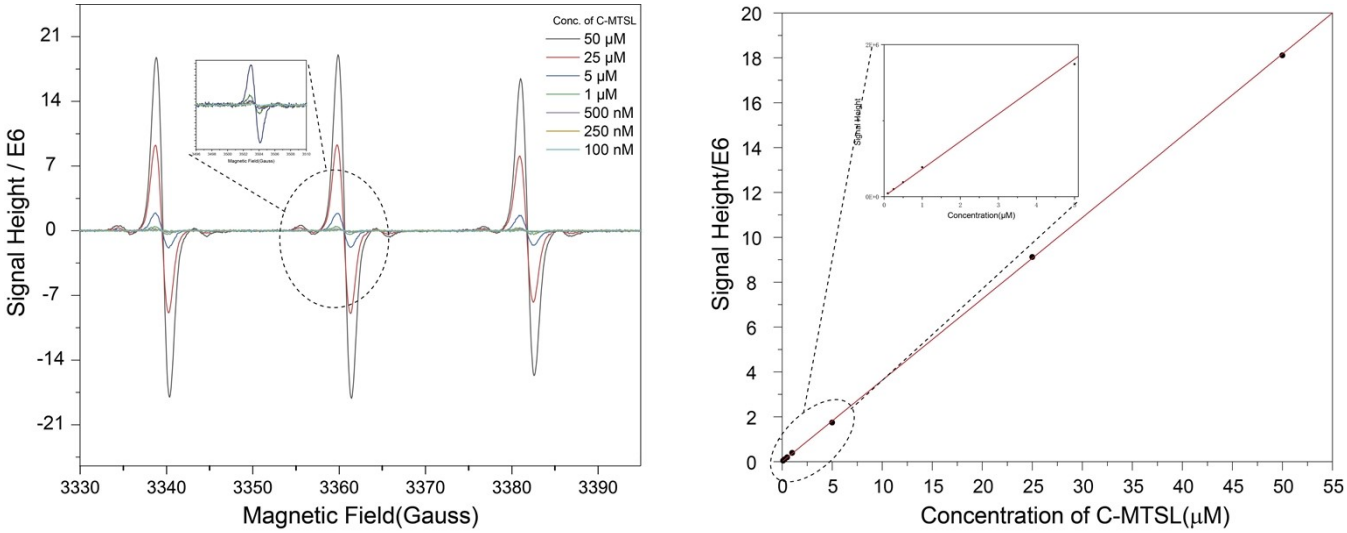

Figure S8. Left panel: EPR spectrum of C-MTSL at different concentrations. The peak height of the central peak was used to calculate then intensity of the signal, which was used for subsequent LOD estimation. Right panel: EPR signal intensity plotted against the concentrations of C-MTSL, fitted by linear least-squares fit.

Next, probe was incubated with different concentrations of caspase-3(8 μM, 14 μM and 20 μM) and EPR signal intensities were measured as a function of time (Figure S9). Then, initial reaction rates were then calculated from the slopes of the EPR intensity versus reaction time at different enzyme concentrations in the linear range (Figure S10). The rate constant was then calculated by linear fitting of the plot of the initial reaction rate versus enzyme concentration. The LOD of caspase-3 was finally estimated using the EPR signal intensity corresponding to the LOD of the released nitroxide divided by the value of the rate constant multiplied with the observation time.

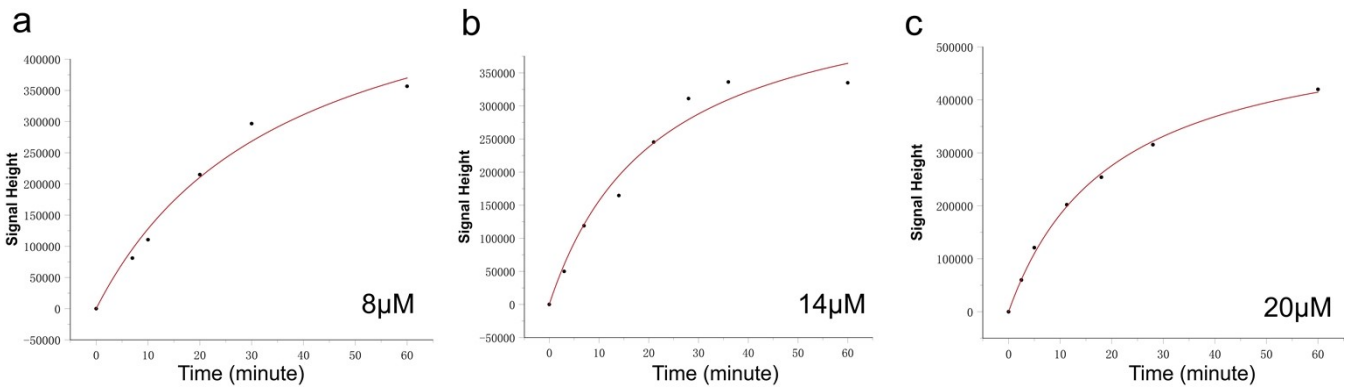

Figure S9. EPR signal intensities(represented by peak-to-peak amplitude of the central peak of EPR spectrum) versus reaction time for reactions with different caspase-3 concentrations(8 μM, 14 μM and 20 μM).

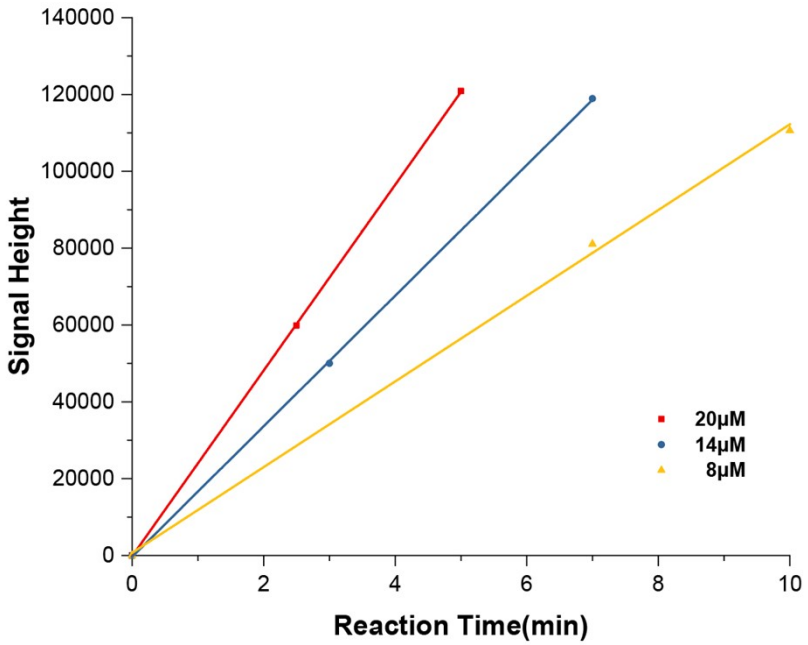

Figure S10. Linear regression of EPR signal intensity with respect to reaction time in the linear region. The reactions with different concentrations of caspase-3 were represented with different colors.

To test whether LOD determined for the EPR method is accurate, we carried out experiments in which the probe was digested by caspase-3 with concentrations slightly above the LOD (116 nM at 60 min and 58 nM at 120 min). EPR spectrum were collected after incubation periods of 60 minutes and 120 minutes using same experimental parameters as those in the manuscript. As shown in Figure S11, for the 60 minutes' group, a concentration of 150 nM (slightly higher than 116 nM) and 240 nM (twice of 116 nM) gave rise to distinguishable EPR peaks. For the 120 minutes' group, a concentration of 80 nM (slightly higher than 58 nM) and120 nM (twice of 58 nM) also gave rise to distinguishable EPR peaks. In particular, the "Control" samples, in which probe was incubated with HEPES buffer instead of caspase-3, displayed no detectable EPR peaks. Therefore, we conclude that the LOD calculated for the EPR assay is reliable.

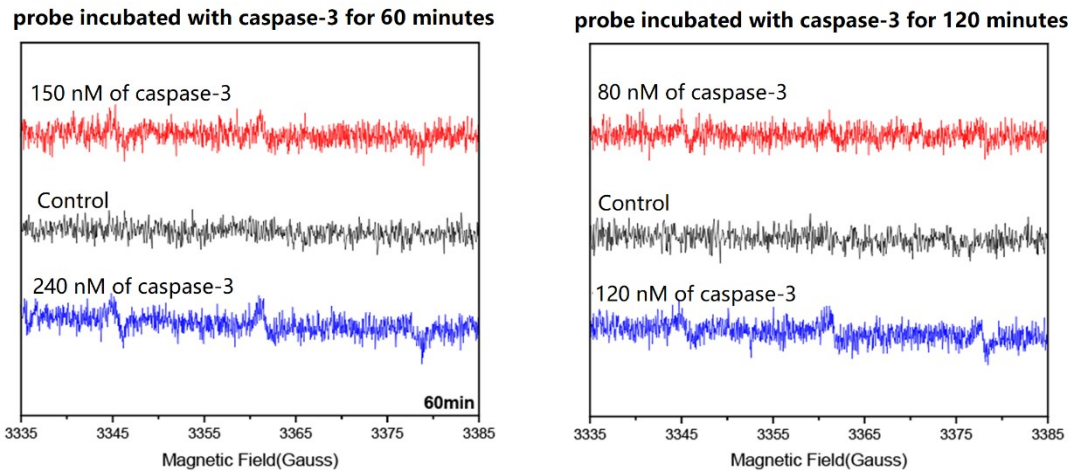

Figure S11. EPR spectrum for the probes digested by caspase-3 with concentrations slightly above the determined LOD.

Cell culture and detection of caspase-3 in cell lysates

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL). The cells were kept in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. For the experimental group, after the cells were cultured for 24 hours, 5 μM staurosporine (STS) was added to the culture medium to induce cell apoptosis for 4 hours.[7,8] The culture medium was discarded and the cells were washed with PBS buffer (pH 7.4) for three times. The cells were then lysate in lysis buffer by sonication, followed by centrifugation to remove the cell debris. Supernatant was collected and utilized for subsequent EPR measurement within 1 hour (stored at ice-water bath). Lysate from HeLa cells without STS treatment was also prepared as control group following the same procedure. Cell lysates were collected and directly treated with 0.5 mg probe for 30 and 60 minutes at 37 ℃. The supernatant was then magnetically separated after incubation and was then subjected to EPR measurement. Additionally, the inhibitory effect of caspase-3 specific inhibitor, AC-DEVD-CHO, was also investigated by adding the inhibitor (final conc. 1 mM) to the reaction mix.

To be noted, the cytotoxicity of the probe in this assay was also tested. The changes in cell viability after incubating with various concentrations of probe was assayed using the Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Kyushu, Japan). In brief, HeLa cells were seeded into a 96-well plate and then treated with probe at different concentrations for 2 hours . Cell viability was assessed based on the optical density at 450 nm. As shown in Figure S12, the probe showed negligible influence on the cell viability even at high concentration (1 mg/ml).

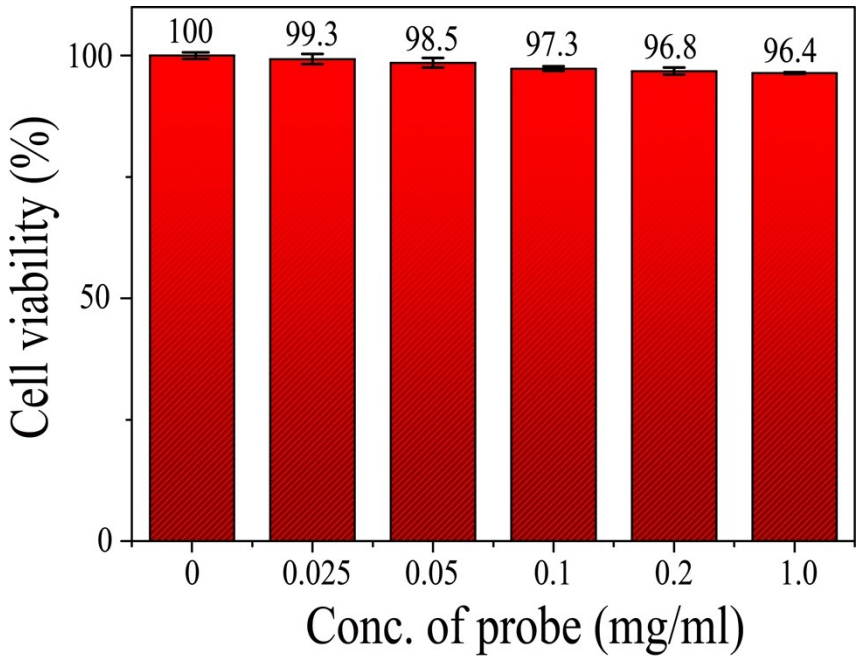

Figure S12. The toxicity effect of the probe on cell viability at different concentrations.

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