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Protease Biosensor by Conversion of a Homogeneous Assay into a Surface-Tethered Electrochemical Analysis Based on Streptavidin–Biotin Interactions

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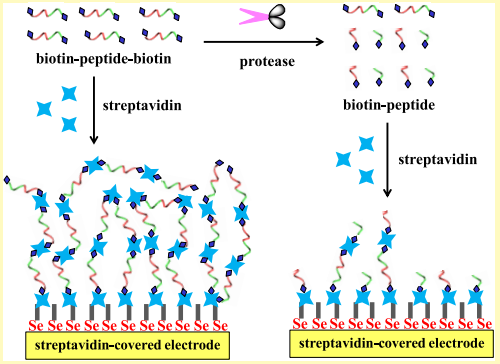
ABSTRACT: This work proposed a new sensing strategy for protease detection by converting a homogeneous assay into a surface-tethered electrochemical analysis. Streptavidin (SA), a tetramer protein, was used as the sensing unit based on the SA–biotin coupling chemistry. Caspase-3 was used as the model analyte, and a biotinylated peptide with a sequence of biotin–GDEVDGK–biotin was designed as the substrate. Specifically, the peptide substrate could induce an assembly of SA to form $(SA–biotin–GDEVDGK–biotin)_n$ aggregates through SA–biotin interactions, which was confirmed by atomic force microscopy (AFM). The peptide substrate-induced assembly of SA was facilely initiated on an electrode–liquid surface by modification of the electrode with SA. The in situ formation of $(SA–biotin–GDEVDGK–biotin)_n$ aggregates created an insulating layer, thus limiting the electron transfer of ferricyanide. Once the peptide substrate was cleaved into two shorter fragments (biotin–GDEVD and GK–biotin) by caspase-3, the resulting products would compete with biotin–GDEVDGK–biotin to bind SA proteins immobilized on the electrode surface and distributed in a solution, thus preventing the in situ formation of $(SA–biotin–GDEVDGK–biotin)_n$ assemblies. With the simple principle of the substrate-induced assembly of SA, a dual-signal amplification was achieved with improved sensitivity. Taking advantage of high sensitivity, simple principle, and easy operation, this method can be augmented to design various surface-tethered biosensors for practical applications.



KEYWORDS: *protease, electrochemical biosensor, caspase-3, surface-tethered analysis, streptavidin*

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Proteases can catalyze the hydrolysis of amide bonds in peptides or proteins. In humans, 1.7% of genes are encoded by proteases. These enzymes are responsible for protein digestion, wound healing, immune system activation, and so on.[1] Proteases have become promising biomarkers and potential therapeutic targets for many diseases, such as cancer, cardiovascular diseases, Alzheimer's disease, human immunodeficiency virus, thrombosis, and diabetes.[2,3] Therefore, a simple, sensitive, and efficient method for protease detection is of great significance for medical diagnostics and therapeutics.

Homogeneous assays, such as high-performance liquid chromatography (HPLC), gel electrophoresis, fluorescence energy resonance transfer (FRET), and mass spectrometry, are the traditional methods for protease detection.[3,4] However, homogeneous assays are sample-consuming, exhibit low sensitivity and poor anti-interference, and/or require the use of complex and expensive instruments. Conversely, heterogeneous assays in which substrate molecules are fixed on a solid surface allow for the simultaneous and multiplexed detection of proteases with improved sensitivity and selectivity. Recently, great efforts have been put into the development of efficient heterogeneous assays for protease detection, including electrochemistry,[5−9] electrochemiluminescence,[10] photoelectrochemistry,[11−13] fluorescence,[14,15] surface-enhanced Raman scattering (SERS),[16] matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS),[17,18] and surface plasmon resonance (SPR).[19] Among these, heterogeneous electrochemical assays have attracted increasingly more attention because of their high sensitivity, simplicity, fast response, and miniaturization. In these methods, a peptide substrate is usually labeled with a redox reporter or biotin group to produce a detectable electrochemical signal.[7,9,20] Moreover, enzymes and/or nanomaterials can be integrated with proteolytic cleavage to improve detection sensitivity.[21−24] Despite the satisfactory achievements, most of the heterogeneous assays are still subject to many limitations. For example, the methods

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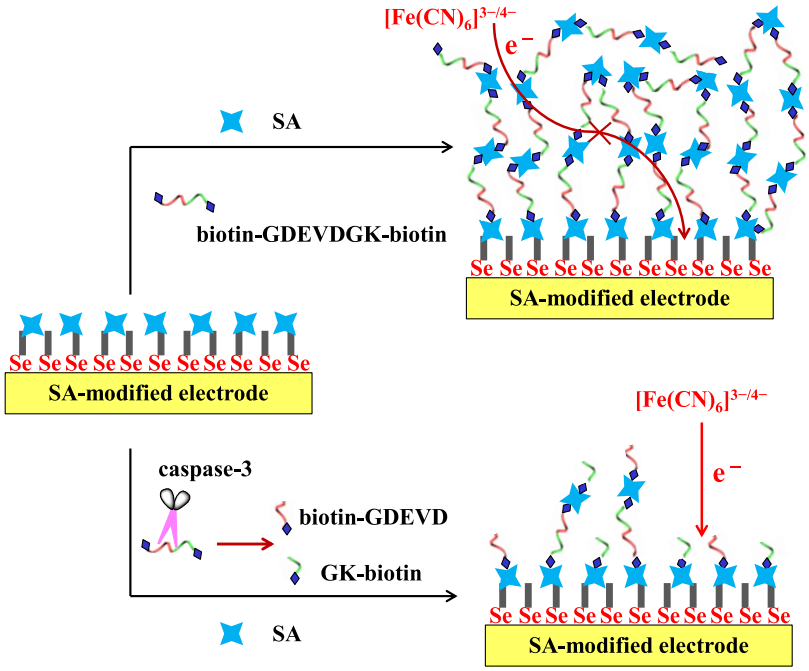
Scheme 1. Schematic Representation of the Surface-Tethered Electrochemical Analysis for Caspase-3 Detection



involving the immobilization of a peptide substrate require an elaborate design of the peptide sequence to ensure that the peptide on the solid surface can be recognized and cleaved by a protease.[25,26] Moreover, an electrode modified with redox-labeled peptides cannot be recycled and may suffer a significant signal loss after long-term preservation.[27] Therefore, it is still desirable to design a simple, sensitive, and general heterogeneous strategy for the detection of various proteases.

A homogeneous assay based on the peptide-induced aggregation of metal nanoparticles is one of the prevalent strategies known for protease detection.[28,29] In this method, cleavage of the peptide substrate into two shorter fragments can induce or prevent the aggregation of metal nanoparticles. Although this method exhibits low sensitivity and poor anti-interference for protease analysis in biological fluids,[30,31] it is advantageous due to its simple manipulation principle and detection procedure. This method brings forth new ideas to us. Herein, we proposed that the aggregation-based homogeneous protease assay can be converted into a signal-enhanced surface-tethered electrochemical analysis, thereby infusing an existing principle into another field. To demonstrate the feasibility and sensitivity of the strategy for protease detection, a proof-of-concept verification experiment was designed and performed with caspase-3 as the model analyte. Electrochemical impedance spectroscopy (EIS), a powerful tool to monitor the change of surface processes and properties, can be used to measure the change of electron transfer resistance. Biomolecules such as proteins and nucleic acids captured by a sensor electrode can prevent the electron transfer of $[Fe(CN)_6]^{3-/4-}$ by creating an insulating layer. In this work, streptavidin (SA), a tetramer protein that can bind to four biotin molecules with high specificity, was used as the signal element and electrode modifier to construct the sensing platform. The principle of this method is illustrated in Scheme 1. SA molecules modified on the electrode surface can capture the peptide substrates (biotin−GDEVDGK−biotin) via SA−biotin interactions. Then, the captured peptide substrates allow the attachment of more SA molecules through the same interaction. As a result, an increasing number of peptide substrates and SA molecules in the solution are circularly immobilized on the electrode surface, leading to the in situ formation of $(SA-biotin-GDEVDGK-biotin)_n$ aggregates. Thus, the substrate-triggered assembly of SA in solution can be readily initiated on the solid−liquid (electrode−electrolyte) surface. The self-assembled $(SA-biotin-GDEVDGK-biotin)_n$ aggregates on the electrode surface can limit the electron transfer and significantly increase the impedance response. Once the peptide substrate was cleaved into two fragments (biotin−GDEVD and GK−biotin) by caspase-3, the substrate-triggered assembly of SA would be prevented. Meanwhile, the released fragments of biotin−GDEVD and GK−biotin can be captured by the SA-modified electrode, thus inhibiting the in situ formation of $(SA-biotin-GDEVDGK-biotin)_n$ aggregates on the electrode surface. On the basis of this concept, a general method is proposed for converting a homogeneous assay into a more sensitive surface-tethered electrochemical analysis by dual-signal amplification. Moreover, the "one-step" immobilization-free method is simple and obviates the need for additional enzymes or nanomaterials for signal amplification.

EXPERIMENTAL SECTION

Chemicals and Reagents. Biotinylated peptide (biotin−GDEVDGK−biotin) was synthesized and purified by China Peptide Co., Ltd. (Shanghai, China). L-Selenocystine was purchased from Red Chemical Technology (Shanghai) Co., Ltd. (Shanghai, China). Recombinant SA, biotin, glucose, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), ethylene glycol-bis-(aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), and tris(hydroxymethyl)aminomethane (Tris) were provided by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Caspase-3, thrombin, *β*-secretase, human serum protein HSA, *β*-amyloid peptide, 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC), and *N*-hydroxysulfosuccinimide (NHS) were provided by Sigma-Aldrich (Shanghai, China). Prostate-specific antigen (PSA) was provided by Linc-Bio Science Co., Ltd, (Shanghai, China). SA-coated magnetic beads were ordered from

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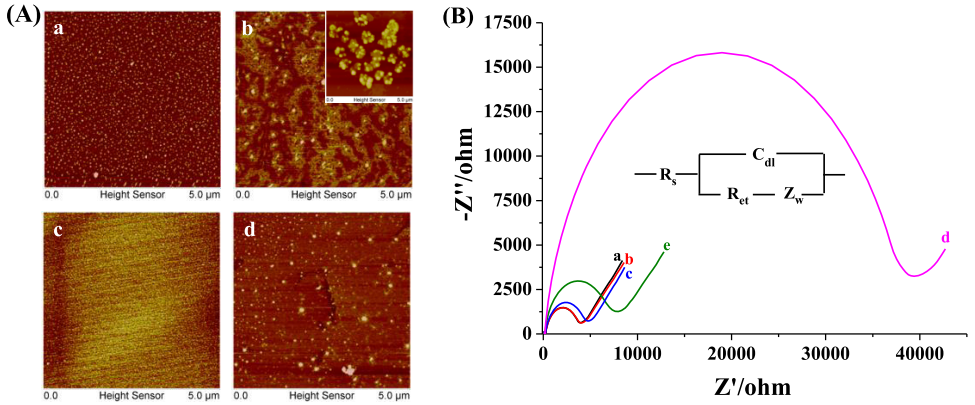

Figure 1. (A) AFM images of SA (panel a), SA/biotin−GDEVDGK−biotin (panel b), biotin−GDEVDGK−biotin (panel c), and SA/biotin/biotin−GDEVDGK−biotin (panel d). Final concentrations of SA, biotin−GDEVDGK−biotin, and biotin are 0.5, 0.5, and 2 μM, respectively. For the inset in panel b, the concentrations of SA and biotin−GDEVDGK−biotin are 0.5 and 1 μM, respectively. (B) EIS responses of the SA-modified electrode before (curve a) and after incubation with SA (curve b), biotin−GDEVDGK−biotin (curve c), and the mixture of SA/biotin−GDEVDGK−biotin (curve d). Curve e corresponds to the SA-modified electrode treated with biotin−DNA−biotin, rinsing buffer, and SA. Final concentrations of SA and biotin−GDEVDGK−biotin are 0.25 and 0.5 μM, respectively.

Thermo Fisher Scientific (Shanghai, China). Staurosporine (STS), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and other chemicals were acquired from Aladdin Reagent Co., Ltd. (Shanghai, China). All of the aqueous solutions were prepared with ultrapure water purified with a Milli-Q purification system.

Characterization of Assemblies. The peptide-induced assemblies of SA were characterized by atomic force microscopy (AFM) (Bruker Nano Inc., Santa Barbara, CA). The SA powder was dissolved in 10 mM phosphate buffer (pH 7.4) to a concentration of 2 μM (~104 μg/mL). The peptide biotin−GDEVDGK−biotin was dissolved in 50 mM NaOH to a concentration of 1 mM and then diluted to a desired concentration with the buffer. Then, 12.5 μL of 2 μM SA, 5 μL of 5 or 10 μM peptide, and 32.5 μL of phosphate buffer were mixed and incubated for 5 min at 37 °C. After that, 10 μL of the mixture was put on a newly peeled mica slide and kept for 30 min. After being rinsed with water, the mica slide was dried under a very gentle nitrogen stream. The surface morphology of the slide was imaged in tapping mode.

Preparation of the Sensor Electrode. In contrast to the Au−S interface, the Au−selenium (Au−Se) interface has a higher bonding energy.[32,33] Thus, the sensing platform with the Au−Se interface shows high stability and fidelity by avoiding the signal distortion induced by biological thiols. In this work, selenocysteine self-assembled monolayers (SAMs) were formed to create an electrode−biomolecule interface. Briefly, a cleaned electrode was first incubated with 100 μM L-selenocystine for 12 h in a pH 7.4 buffer solution (10 mM phosphate and 1 mM TCEP) to form the selenocysteine SAMs. Then, the electrode was rinsed with ultrapure water and activated by a mixture of 0.2 M EDC and 0.1 M NHS for 15 min. After being washed with water again, the electrode was incubated with 10 μg/mL SA for 4 h. The SA molecules were immobilized on the electrode surface through the amine coupling reaction. The unreacted activated carboxyl groups on the SAMs were blocked by incubating the electrode with 1 mM ethanolamine for 30 min. Finally, the SA-covered sensor electrode was thoroughly rinsed with ultrapure water before use.

Electrochemical Detection of Caspase-3. The peptide substrate (biotin−GDEVDGK−biotin) was first incubated with a given concentration of caspase-3 for 2 h in 20 μL of HEPES buffer (50 mM, pH 7.4) at 37 °C.[34] Then, the SA-covered electrode was incubated with the sample for 5 min, followed by addition of 20 μL of SA solution to the mixture. After incubating again for 30 min, the electrode was thoroughly rinsed with the buffer and ultrapure water. Then, the electrochemical data were collected on a CHI 660 electrochemical workstation (CH Instruments, Shanghai, China) with a Pt auxiliary electrode and a Ag/AgCl reference electrode. The differential pulse voltammograms (DPV) and electrochemical impedance spectroscopy (EIS) responses were recorded in a solution of 5 mM $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{3-/4-}$ (1:1) containing 0.1 M KCl, respectively.

Analysis of Cell Apoptosis. The procedures for the cell culture and extraction followed previous reports. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and kept at 37 °C in a humidified atmosphere (95% air and 5% CO_2). After being cultured for 5 days, the DMEM solution was replaced by a drug-free or a drug-containing medium. After incubation for 8 h, the cells were collected by trypsinization, washed with HEPES buffer, and then lysed with the lysis buffer (10 mM Tris−HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.5% CHAPS, and 10% glycerol).[10,35] The resulting lysates were collected and centrifuged at 12 000 rpm for 10 min. One milliliter of the supernatant was treated with 50 μg/mL SA-coated magnetic beads to eliminate the interference of biotin and then diluted to different multiples for analysis. The caspase-3 activity was detected following the aforementioned procedures.

RESULTS AND DISCUSSION

SA is a tetramer protein with a size of ~66 kDa. The protein can bind to four biotin molecules with a dissociation constant of 10^{-14} M. AFM is a useful tool for direct characterization of microstructures of nanomaterials and biological macromolecules. To demonstrate that biotin−GDEVDGK−biotin can induce the assembly of SA molecules, the morphology of SA in the absence and presence of biotin−GDEVDGK−biotin was characterized by AFM. Figure 1A shows the AFM topography images of a mica surface coated with different samples. The monodisperse SA proteins with a height of 1−2 nm in agreement with a previous report were observed on the mica surface (panel a).[36] The width was significantly larger than the height, owing to the tip dilation effect.[37,38] Addition of biotin−GDEVDGK−biotin to the SA solution resulted in the formation of assemblies (panel b). Note that no particles were observed on the mica surface treated with biotin−GDEVDGK−biotin (panel c); thus, the aggregates should be attributed to the $(SA−biotin−GDEVDGK−biotin)_n$ assemblies. To confirm that the SA assembly was trigged by biotin−GDEVDGK−biotin, the sample composed of SA, biotin, and biotin−GDEVDGK−biotin was characterized by TEM (panel d). As a result, fewer aggregates were observed in comparison to those in the absence of biotin (panel b). This result

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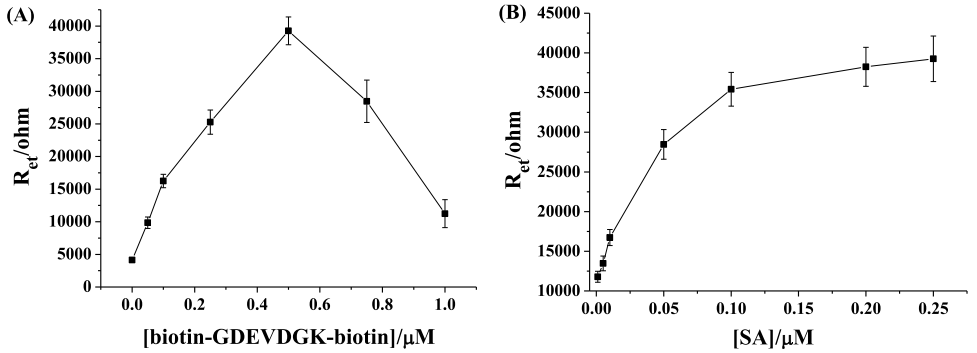

Figure 2. Dependence of R_{et} on the concentration of biotin−GDEVDGK−biotin (A) and SA (B). In panel (A), the final concentration of SA is 0.25 μM. In panel (B), the concentration ratio of biotin−GDEVDGK−biotin to SA is maintained at 2:1.

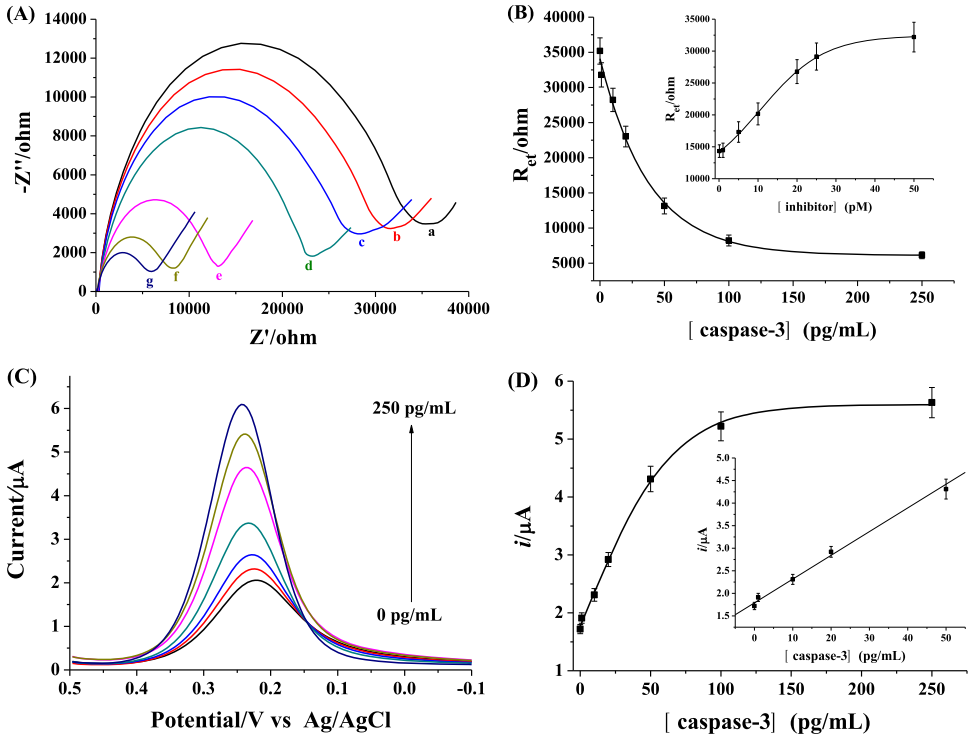

Figure 3. (A) EIS responses for assays of different concentrations of caspase-3 (curve a−f: 0, 1, 10, 20, 50, 100, and 250 pg/mL). (B) Dependence of R_{et} on caspase-3 and inhibitor concentration. DPV responses (C) and calibration curves (D) for the detection of various concentrations of caspase-3. The inset in panel (D) shows the linear segment of the calibration curve.

demonstrated that binding of biotin to SA prevented the formation of $(SA−biotin−GDEVDGK−biotin)_n$ assemblies.

An SA-functionalized electrode was used as the sensor interface. The assembly of SA and biotin−GDEVDGK−biotin on the electrode surface was monitored by EIS. The impedance data were measured by a Randles electrical equivalent circuit including the ohmic electrolyte resistance (R_s), the Warburg impedance (Z_w), the double-layer capacitance (C_{dl}), and the electron transfer resistance (R_{et}). As shown in Figure 1B, no significant enhancement of R_{et} was observed when the SA-modified electrode was incubated with SA (cf. curves a and b) or biotin−GDEVDGK−biotin (curve c) alone. The result indicated that SA was not captured by the SA-modified electrode and the small biotinylated peptide did not significantly limit the electron transfer. However, after being incubated with the SA/biotin−GDEVDGK−biotin mixture, the sensor electrode exhibited a very high R_{et} (curve d). The value was remarkably higher than that obtained by treating the SA-modified electrode with biotin−DNA−biotin, rinsing buffer, and SA (curve e). This suggested that the in situ formed $(SA−biotin−GDEVDGK−biotin)_n$ assemblies can prevent $[Fe(CN)_6]^{3-/4-}$ from entering onto the electrode surface more effectively than the single SA−biotin−GDEVDGK−biotin conjugate, thereby significantly improving the impedance signal.

In this method, biotin−GDEVDGK−biotin played a dual role—as an enzyme substrate and as a cross-linking reagent for the SA assembly. Thus, the concentration ratio of biotin−GDEVDGK−biotin to SA ([biotin−GDEVDGK−biotin]/[SA]) was crucial for the in situ formation of $(SA−biotin−GDEVDGK−biotin)_n$ aggregates on the electrode surface. The dependence of R_{et} on the biotin−GDEVDGK−biotin concentration at a fixed concentration of SA was investigated first. It was found that R_{et} increased with increasing biotin−GDEVDGK−biotin concentration below 0.5 μM (Figure 2A). Then, R_{et} began to decrease dramatically. The result is

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understandable since the binding sites of SA can be saturated by excessive biotin−GDEVDGK−biotin, thus hindering the assembly of tetramer SA proteins and limiting the in situ formation of (SA−biotin−GDEVDGK−biotin)$_n$ aggregates on the electrode surface. Then, the dependence of R_{et} on the SA concentration was investigated. It can be observed that R_{et} increased with increasing SA concentration (Figure 2B). To obtain high sensitivity and a wide linear range, 0.2 μM biotin−GDEVDGK−biotin and 0.1 μM SA were used for caspase-3 detection in the following quantitative analysis.

Under the optimized experimental conditions, caspase-3 at different concentrations was analyzed. As shown in Figure 3A,B, R_{et} decreased with increasing caspase-3 concentration (0−250 pg/mL). The result suggested that higher concentration of caspase-3 can catalyze the hydrolysis of many more peptide substrates. Then, the resulting biotin−GDEVD and GK−biotin fragments competed with biotin−GDEVDGK−biotin to bind SA molecules both on the electrode surface and in solution, thus limiting the formation of (SA−biotin−GDEVDGK−biotin)$_n$ aggregates and producing a low impedance signal. To further confirm that the impedance change is caused by the enzymatic hydrolysis reaction, caspase-3 was preincubated with a well-known inhibitor (DEVD-FMK) and then analyzed. As a result, R_{et} increased gradually with increasing inhibitor concentration (see the inset of Figure 3B). Based on the inhibition efficiency, the half-maximum inhibition value (IC_{50}) for 50 pg/mL caspase-3 was found to be 13 pM. The result is in agreement with a previous report,[39] indicating that the method can be used to evaluate the inhibition efficiency and screen potential inhibitor drugs.

In contrast to the "signal-off" sensing platforms, "signal-on" biosensors exhibit higher sensitivity because of the lower background signal. In addition to EIS, the electron transfer of ferricyanide on the sensing interface has been monitored by differential pulse voltammetry (DPV). It was found that the formation of (SA−biotin−GDEVDGK−biotin)$_n$ aggregates on the electrode surface prevented the electrochemical reduction of $[Fe(CN)_6]^{3-}$. However, cleavage of the peptide substrate by caspase-3 prevented the assembly of SA on the electrode surface, thus promoting the electrochemical reduction of $[Fe(CN)_6]^{3-}$ and allowing for the signal-on detection of caspase-3. As depicted in Figure 3C, the DPV signal increased with the increase of caspase-3 concentration. The method exhibits a good linear relationship for caspase-3 detection in the concentration range from 0 to 50 pg/mL (Figure 3D). The linear equation can be expressed as I (μA) = 0.05[caspase-3] (pg/mL) + 1.79. The detection limit was calculated to be 0.2 pg/mL based on the slope and the standard deviation of the blank response. The value was lower than those obtained by the homogeneous assays, including fluorescence (0.128 ng/mL),[34] colorimetry (5 ng/mL),[40] electrochemistry (27.4 ng/mL),[41] and mass spectrometry (3.02 ng/mL).[26] The detection limit is comparable to or even lower than that achieved by the heterogeneous electrochemical analysis with the signal amplification of enzymes or nanomaterials, such as methylene blue/graphene oxide (0.06 pg/mL),[42] calixarene-functionalized reduced graphene oxide (0.0167 pg/mL),[43] citrate-stabilized (0.1 pg/mL) or CB[8]-capped (24.62 pg/mL) silver nanoparticles,[44,45] magnetic bead/horseradish peroxidase (MB-HRP) (27.4 ng/mL),[46] and gold nanoparticle-coated silica-based mesoporous materials (AuNPs-MCM)/MB-HRP (1.37 pg/mL).[47] The high sensitivity can be attributed to the poor conductivity of (SA−biotin−GDEVDGK−biotin)$_n$ aggregates and the competition reaction between the hydrolysis products (biotin−GDEVD or GK−biotin) and the peptide substrate to bind SA both on the electrode surface and in solution. More importantly, a novel concept was proposed in this work to convert a homogeneous assay into a surface-tethered electrochemical analysis with improved sensitivity.

To demonstrate the specificity of the method, we challenged the biosensor with other biological molecules, including serum protein HSA, proteases (thrombin and PSA), β-amyloid peptide Aβ40, and glucose. As shown in Figure 4, the tested

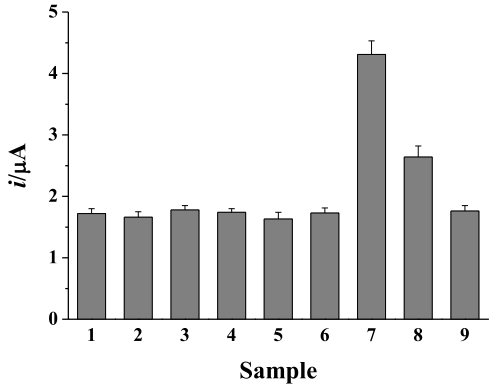

Figure 4. Selectivity of the method to blank control (bar 1), 10 ng/mL HSA (bar 2), 10 ng/mL thrombin (bar 3), 10 ng/mL PSA (bar 4), 1 μM Aβ40 (bar 5), 2 mM glucose (bar 6), 50 pg/mL caspase-3 (bar 7), and 1 nM biotin before (bar 8) and after (bar 9) treatment with SA-coated magnetic beads.

biomolecules did not cause a significant increase in the current (bar 1−6), indicating that the method is highly specific toward caspase-3 detection. The good selectivity can be attributed to the highly specific recognition of caspase-3 to the peptide substrate and the use of the antifouling SA-modified sensor interface. Note that biotin can bind to SA and prevent the formation of (SA−biotin−GDEVDGK−biotin)$_n$ aggregates; application of the biosensor for the direct analysis of a real sample may be limited by the presence of sub-nanomolar biotin.[48,49] Fortunately, it was found that the interference can be well eliminated by removing the biotin species using the commercial SA-coated magnetic beads (bars 8 and 9). To eliminate the interference from biomolecules that may bind to the biotin tag, the sample can be pretreated by addition of free biotin and then the excess biotin molecules can be removed by the magnetic separation. Additionally, the method is very sensitive; thus, the possible interference from other components in biological samples at a high concentration may be eliminated by multiple dilutions.

Monitoring cell apoptosis is of considerable significance in clinical investigation and apoptosis-targeted therapy. Caspase-3 is an activated cysteine protease that plays an important role in intrinsic and extrinsic apoptotic pathways. Therefore, caspase-3 has been recognized as a reliable molecular biomarker and therapeutic target for apoptosis-related diseases.[50] To demonstrate the application of the biosensor for apoptosis evaluation, caspase-3 in living and apoptotic MCF-7 cells was analyzed. STS, a well-known apoptosis inducer, was used to trigger the cell apoptosis. The apoptosis was confirmed by observing the cell morphology (Figure 5A). The lysates from living and apoptotic MCF-7 cells were collected and diluted by different multiples for analysis. As shown in Figure 5B, no significant

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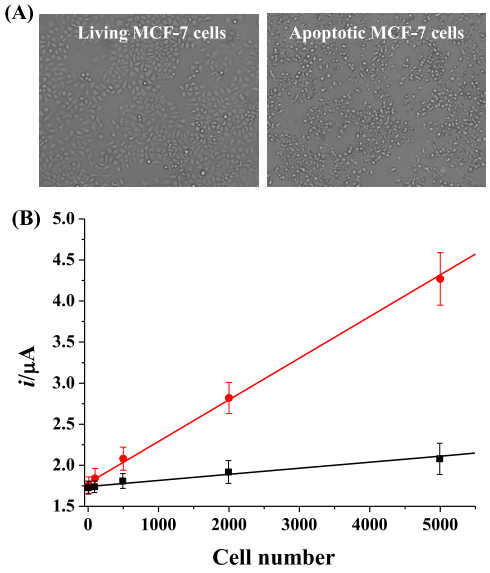

Figure 5. (A) Confocal images of living and apoptotic MCF-7 cells. (B) Dependence of peak current on the number of living (black curve) and apoptotic (red curve) cells.

increase in the peak current was observed for the increasing concentration of living cells. However, the current increased significantly with increasing concentration of apoptotic cells. The result suggested that caspase-3 was activated during apoptosis. Thus, the biosensor exhibits great potential to evaluate cell apoptosis and screen therapeutic drugs for apoptosis-related diseases.

CONCLUSIONS

In summary, a protease biosensor was developed by converting a homogeneous assay into a surface-tethered electrochemical analysis. A tetramer SA was used as the sensing element, and an SA-modified electrode was used as the sensor interface. In contrast to the reported heterogeneous strategies for protease detection, the method did not require the immobilization of peptides on a solid surface, thus facilitating the cleavage of peptide substrate by a protease. With caspase-3 as a model analyte, the biosensor exhibited high sensitivity and specificity and was used for evaluation of cell apoptosis with satisfactory results. The detection limit of 0.2 pg/mL was significantly lower than those of the homogeneous assays and was comparable to that of the heterogeneous electrochemical analysis with the signal amplification of enzymes or nanomaterials. Since biotin can be readily modified on peptides and nucleic acids, other surface-tethered sensing platforms can be developed to detect various analytes (e.g., DNA, microRNAs, and nucleases) by converting a homogeneous assay into a sensitive surface-tethered analysis based on this approach.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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