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Short review

Electrochemical Biosensors for Probing of Protease Activity and Screening of Protease Inhibitors

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Protease is one of the most important physiological enzymes because proteolytic processing is the final step in the expression of the activity of a great variety of proteins. Standard assays for proteases include those based on radioisotopes or on fluorogenic substrates. In recent years, electrochemical biosensors have been shown to be a promising alternative to mass- and fluorescence-based sensors for the specific detection of protein, DNA and small biomolecules in view of their high sensitivity, simplicity, rapid response, and compatibility with miniaturization. In this review, we summarized the strategies for development of electrochemical biosensors to detect proteases and the inhibitors.

Keywords: Protease; Inhibitors; Electrochemical biosensors; Redox tags; Label-free; Signal amplification

1. INTRODUCTION

Protease catalyzes the hydrolytic degradation of proteins to peptides and plays critical roles in many fundamental biological processes, such as cell differentiation and growth, blood coagulation, angiogenesis, wound healing, apoptosis, and lipid metabolism [1,2]. Determination of the concentration level and activity of protease is important for the screening of protease inhibitors and pathologies associated with its presence, and for the development of effective and selective therapeutics. Traditional methods for protease detection include high-performance liquidchromatography (HPLC), fluorescence, enzyme-linked immunosorbent assay (ELISA) and gel electrophoresis. However, these methods are usually time-consuming, lack sensitivity and/or require

complicated instruments [3]. Recently, electrochemical biosensors have attracted widespread interest in view of their high sensitivity, simplicity, rapid response, and compatibility with miniaturization. In this work, we introduced the method and principle of the electrochemical protease biosensors, and evaluated their analytical merits in detection of protease and screening of the inhibitors.

2. DETECTION METHODS

2.1 Redox-labeled peptide probes

Among kinds of electrochemical protease biosensors, redox tags (most commonly methylene blue or MB and ferrocene or Fc) labeled peptide probes site-specifically attached to an interrogating electrode is one of the most attractive approaches. Protease-mediated change in the number or configuration of redox tags leads to a readily detectable change in Faradaic current. The current progress in the design and utilization of redox-labeled peptide probes is discussed herein.

2.1.1 Protease-mediated cleavage of redox-labeled peptides

Since its discovery over 50 years ago, Fc and its derivatives have been used as the redox markers in electrochemical measurements because of its well-defined and reversible redox reaction. Peptides modified with Fc moieties have also been successfully used for the electrochemical analysis of proteases on gold electrodes. For example, matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play a major role on cell behaviors including cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense. Change in the concentration level of MMPs has been associated with some diseases. Lin and co-workers are the first to develop electrochemical proteolytic beacon (EBP) biosensors using Fc-peptides modified gold electrodes for the detection of MMPs [4]. As shown in Fig. 1, the peptide RPLALWRSC labeled with Fc was first immobilized onto a gold electrode. The electrode showed a redox signal based on the Fc moieties. After treatment of this electrode with MMPs, its electrochemical signal was decreased in proportion to an increase of the amount of MMPs. Lately, the same approach was extended by severer groups for the assay of other proteases [5-8]. For example, caspases are a class of cysteine-dependent, aspartate-specific proteinases related to cellular apoptosis. Xiao et al. prepared an Fc-GDGDEVDGC modified gold electrode and reported its application in detection of caspase activity [5,9]. Plasmin is a trypsin-like serine protease that acts to dissolve fibrin blood clots into soluble products and plays important roles in tissue proliferation, cellular adhesion, pathogen invasion, cancer invasion, and metastasis. Ohtsuka et al. described the electrochemical assay of plasmin activity and its kinetic analysis on Fc-labeled peptide (Fc-Lys-Thr-Phe-Lys-Gly-Gly-Gly-Gly-Gly-Gly-Cys-NH_2) modified gold electrode [6]. The detection limit for plasmin was as low as 50 ng/ml or 0.15 mU/ml. Moreover, Demaille and co-workers have described how the design of electrode-supported Fc-peptide sensing systems can be improved for these systems to be used as powerful tools for studying the kinetics of enzyme action toward surface-attached peptides [9].

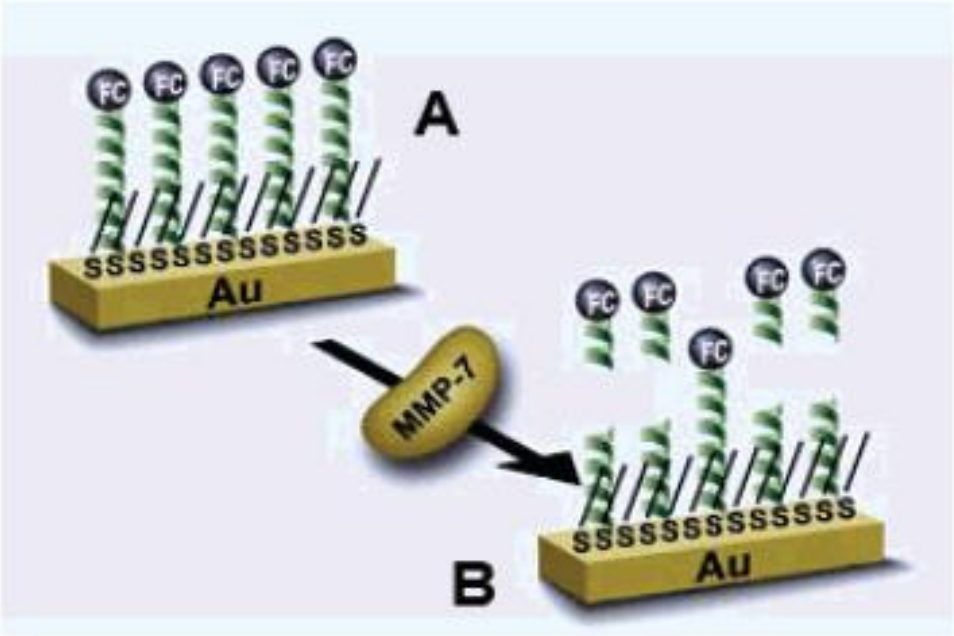

Figure 1. Schematic of EPB for detection of matrix metalloproteinase: (A) self-assembling electrochemical Fc-peptide conjugate on the gold substrate; (B) cleavage of EPB in the presence of MMP-7. Reprinted with permission from [4]. Copyright 2006 American Chemical Society.

The detection sensitivity can be enhanced by reducing the electrode size. Well separated nanoelectrode arrays (NEAs), either in regular pattern or in random distributions (also referred to as nanoelectrode ensembles (NEEs)), have attracted extensive interests for highly sensitive electroanalysis, measuring fast electrochemical kinetics, and biosensing [10]. Typically, carbon-based NEAs are particularly attractive for biosensors due to the wider applicable potential window and ease in biofunctionalization through robust covalent attachment [11]. For this consideration, Swisher et al. reported the detection of protease activity using nanoelectrode arrays (NEAs) fabricated with vertically aligned carbon nanofibers (VACNFs) (Fig. 2) [12]. Two types of tetrapeptides specific to cancer-mediated proteases legumain and cathepsin B are covalently attached to the exposed VACNF tip, with a Fc moiety linked at the distal end. When supplied with the specific protease, the peptide is expected to be cleaved at the particular site, leading to the release of the Fc moiety from the electrode surface into the bulk solution and decrease in the Fc redox signal.

Besides Fc derivatives, MB derivatives are also good redox tags for labeling of peptide and DNA probes. Revzin's group reported the electrochemical detection of MMP9 activity using MB-labeled peptide probes on microfabricated 300 μm diameter Au electrodes (Fig. 3) [13]. As a result, incubating the electrodes with MMP9 solutions resulted in the cleavage of the peptide fragment and a decrease in the electrochemical signal. The detection limit of this method was 60 pM with a linear range extending to 50 nM.

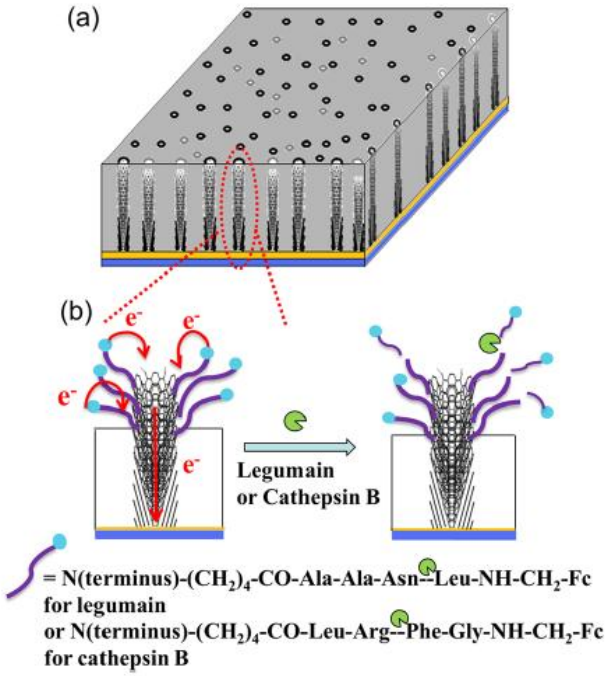

Figure 2. Schematic Diagram of the Cleavage of Fc-Linked Tetrapeptides at the VACNF NEA Tip by Specific Proteases. (a) A random VACNF array embedded in the SiO_2 matrix. (b) Electron transfer from appended ferrocene at the distal end of the peptide to the underlying metal film electrode through the VACNFs and the loss of the electrochemical signal from ferrocene due to the cleavage of the peptide at specific sites. Reprinted with permission from [12]. Copyright 2013 American Chemical Society.

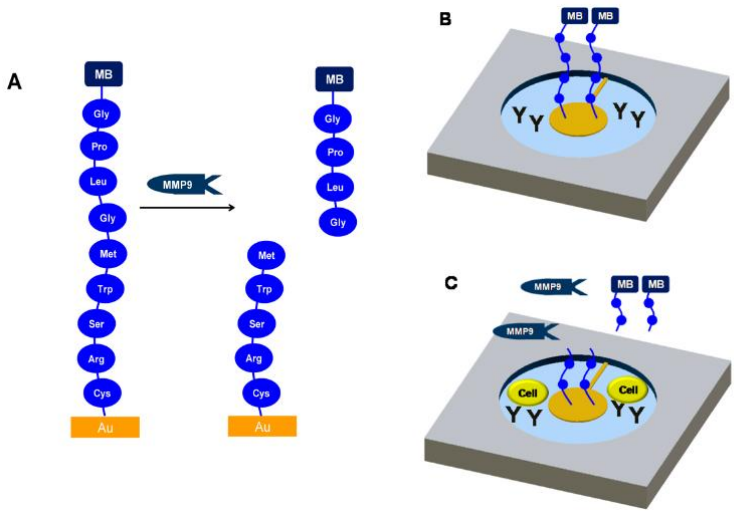

Figure 3. Detection of MMP9 release from monocytes. (A) Peptide Gly-Pro-Leu-Gly-Met-Trp-Ser-Arg-Cys is assembled on Au surface and is subject to MMP9 mediated cleavage between Gly and Met. (B) An individual cell sensing site, consisting of an electrode with redox-labeled peptide, Abs for cell capture and nonfouling PEG hydrogel. (C) Monocytes become captured on Ab-modified regions and release protease molecules upon activation. Reduction current decreases upon cleavage of peptide and diffusion of fragments carrying redox labels. Reprinted with permission from [13]. Copyright 2013 American Chemical Society.

Moreover, Liu's group developed a simple and sensitive electrochemical method for the detection of thrombin activity and inhibition by taking advantage of an electro-labeled tripeptide substrate (H-D-Pro-Phe-Arg-p-aminodiphenylamine) [14], which is immobilized on gold electrode surface and can be specifically recognized and cleaved at the amide group connecting arginine and p-aminodiphenylamine (pADA) by thrombin. Consequently, thrombin activity can be conveniently monitored by measuring the electrochemical signals ascribed to the remaining pADA as the electroactive reporter on electrode surface. Also, Inoue et al. reported the amperometric detection of protease using a p-aminophenol (p-AP) conjugated substrate Boc-Leu-Gly-Arg-pAP (LGR-pAP) on a screen-printed electrode chip [15]. The clotting protease enzyme is activated by an endotoxin-induced Limulus amebocyte lysate (LAL) cascade reaction. The endotoxin activated zymogens contained in the LAL to generate p-AP, which was then electrochemically detected by potential step chronoamperometry. The observed oxidation current increased with the endotoxin concentration ranging from 10 to 1000 EU L^{-1}.

2.1.2 Interaction of protease and redox-labeled peptide probes

The interaction of the surface with the biomolecule will alter the electron-transfer properties of the systems. Kraatz's group reported several proteases biosensors by employing Fc-labeled recognition peptides on gold electrodes [16-20]. Specifically, they first reported an electrochemical approach to detect HIV-1 protease on gold electrode modified with Fc-conjugated pepstatin [17,18]. When HIV-1 protease was binding to pepstatin and encapsulating the Fc redox center on the surface, the formal potential for the surface-bound Fc-conjugated pepstatin shifted to higher potentials, indicating that the oxidation of the Fc group was becoming increasingly more difficult. By reasonably designing the sequence of peptide, they also demonstrated the application of the method in the detection of papain and hepatitis C viral NS3-4A protease [21].

Integration of metallic nanoparticles with carbon nanotubes (CNTs) has attracted much interest because of the low resistance ohmic contacts of these composites. Of particular interest is the attachment of AuNPs to the side walls of CNTs to fabricate highly efficient sensor devices. CNT and gold nanoparticles (AuNPs)-modified electrodes have many advantages such as high surface area, favorable electronic properties, ease of biomolecule attachment, and electrocatalytic effects [22]. For these views, Luong's group reported the detection of HIV-1 protease and the screening of its inhibitor on Fc-pepstatin-assembled thiolated single-walled carbon nanotube (SWCNT)/AuNPs modified gold electrodes (Fig. 4) [22,23]. As a result, HIV-1 protease at the picomolar level was readily detected by electrochemical impedance spectroscopy (EIS) because of the specific binding of this enzyme to Fc-labeled pepstatin. The value is lower than that obtained at the gold electrode (4 nM) [16].

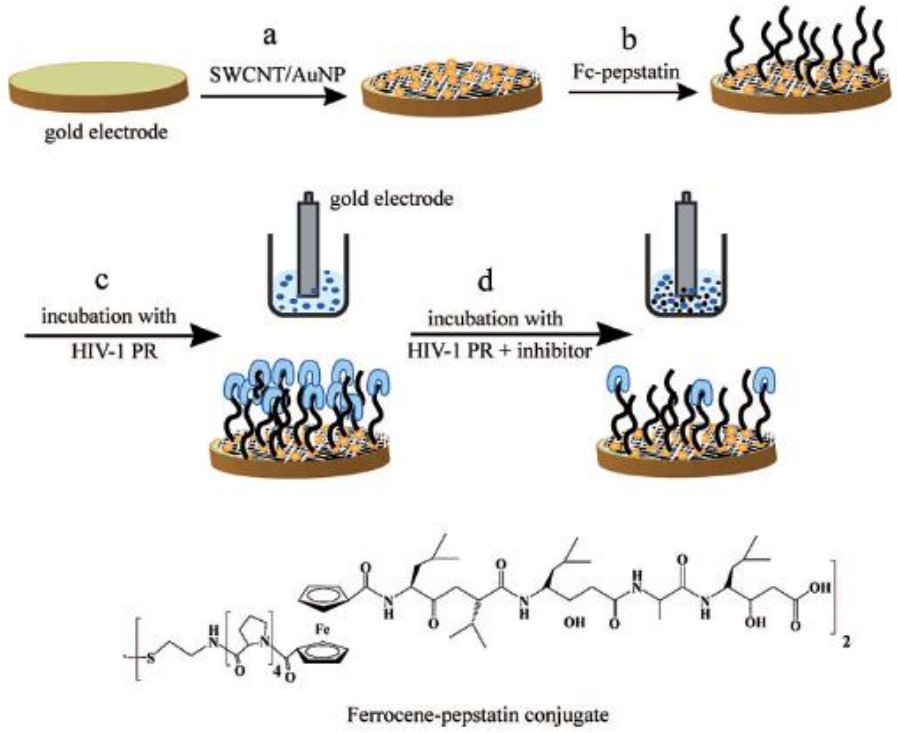

Figure 4. Schematic illustration for the preparation of ferrocene-pepstatin conjugate/thiolated single-walled carbon nanotube (SWCNT) and gold nanoparticle (AuNP) modified electrodes and their use for detecting HIV-1 protease and the subsequent assay of HIV-1 protease inhibitors. Reprinted with permission from [23]. Copyright 2008 American Chemical Society.

2.2 Label-free strategies

The detection of biomolecular interactions by electrochemical methods requires in general the presence of a redox-active probe as part of the detection system. The redox probe can be in solution or can be covalently attached to the molecule capturing the biomolecule from solution. In both cases, the interaction of the surface with the biomolecule will alter the electron-transfer properties of the systems. Labeling of peptide with Fc tags mentioned above offers better sensitivity. However, Fc-labeled sensor exhibits significant signal loss after multiple regeneration/assay cycles and long-term storage due to the degeneration of Fc tags [24]. Moreover, the labeling process would make the experiments complex and influence the bioaffinity between the probes and their targets. Therefore, there has been interest in developing label-free and low cost biosensors in recent years. Aptamers are artificial nucleic acid ligands, selected through combinatorial libraries to bind specifically target molecules. Rodriguez et al. demonstrated that the interaction of the target protein (e. g. lysozyme) and the aptamer probe caused the increase in the electrochemical impedance of anti-lysozyme aptamer probe modified gold electrode in the $[Fe(CN)_6]^{3-/4-}$ solution [25]. Furthermore, Cao et al. developed a simple electrochemical method for assay of protease (trypsin) activity on unmodified-peptides covered gold electrode [26]. As shown in Fig. 5, cationic residue incorporated at the N-terminal forms positively charged monolayer, blocking the penetration of positively charged $[Ru(NH_3)_5Cl]^{2+}$. When the peptide is cleaved by the target protease, a peptide fragment containing the cationic residue will be removed from the electrode, which

accordingly weakens the blocking effect and resulting in an observed well-defined signal from the redox of $[Ru(NH_3)_5Cl]^{2+}$. With the same principle, Deng et al. reported a label-free electrochemical biosensor for detection of active prostate specific antigen (PSA, a serine protease produced by both normal prostate glandular cells and prostate cancer cells) [27]. In the work, peptide with a sequence of CHSSKLQK was immobilized onto gold electrode. The positively charged lysine residues facilitated the access of the negatively charged $[Fe(CN)6]^{3-/4-}$ probes to the electrode surface. The cleavage of peptide by PSA will induce loss of the positively charged lysine residues, leading to neutral SAMs that form a barrier for the electron transfer between redox probe and electrode. As a result, the decrease in the current of $[Fe(CN)_6]^{3-/4-}$ is linearly proportional to the increase of PSA concentration in the range of 1 ~ 60 ng mL^{-1} with a detection of 1~60 ng mL^{-1}.

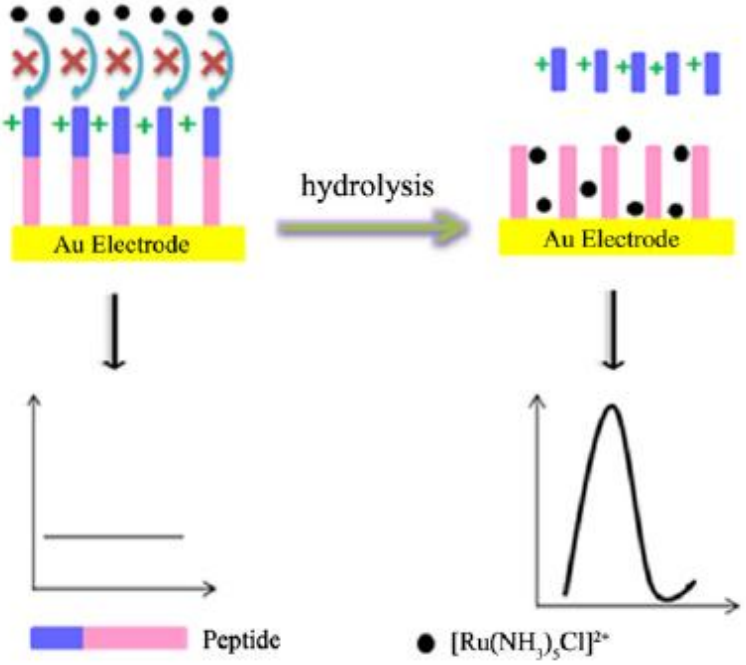

Figure 5. Schematic illustration of the mechanism to develop a simple and generalized electrochemical method for monitoring the activity of protease. Reprinted with permission from [26]. Copyright 2013 Elsevier.

Moreover, Li et al. reported a novel method with electrochemical technique to detect various proteases [28]. In this strategy, the working electrode is modified with the peptide probes for the target proteases. As shown in Fig. 6, the peptide probe is composed of three motifs: (1) located at one end of the probe is the absorption-resistant sequence serving to minimize nonspecific interaction with the non-target protein and to immobilize the probe onto the surface of the working electrode, (2) at the other end is the seed peptide sequence to initiate and accelerate amyloid-beta misfolding on the electrode surface, and (3) between these two motifs is the substrate sequence of the target protein, i.e., protease. If the substrate peptides were cut by proteases, the distal seed peptide will be removed from the electrode surface. In the absence of proteases, the seed peptides can initiate and accelerate amyloid-beta misfolding on the electrode surface. Consequently, the formed aggregates strongly block the

electron transfer of the in-solution electroactive species with the electrode, resulting in suppressed signal readout. Nevertheless, in the presence of proteases, enzyme cleavage may lead to greatly mitigated protein misfolding and evident signal enhancement. Since the contrast in signal readout between the two cases can be amplified by using the protein misfolding step, high sensitivity suitable for direct detection of proteases in serum can be achieved.

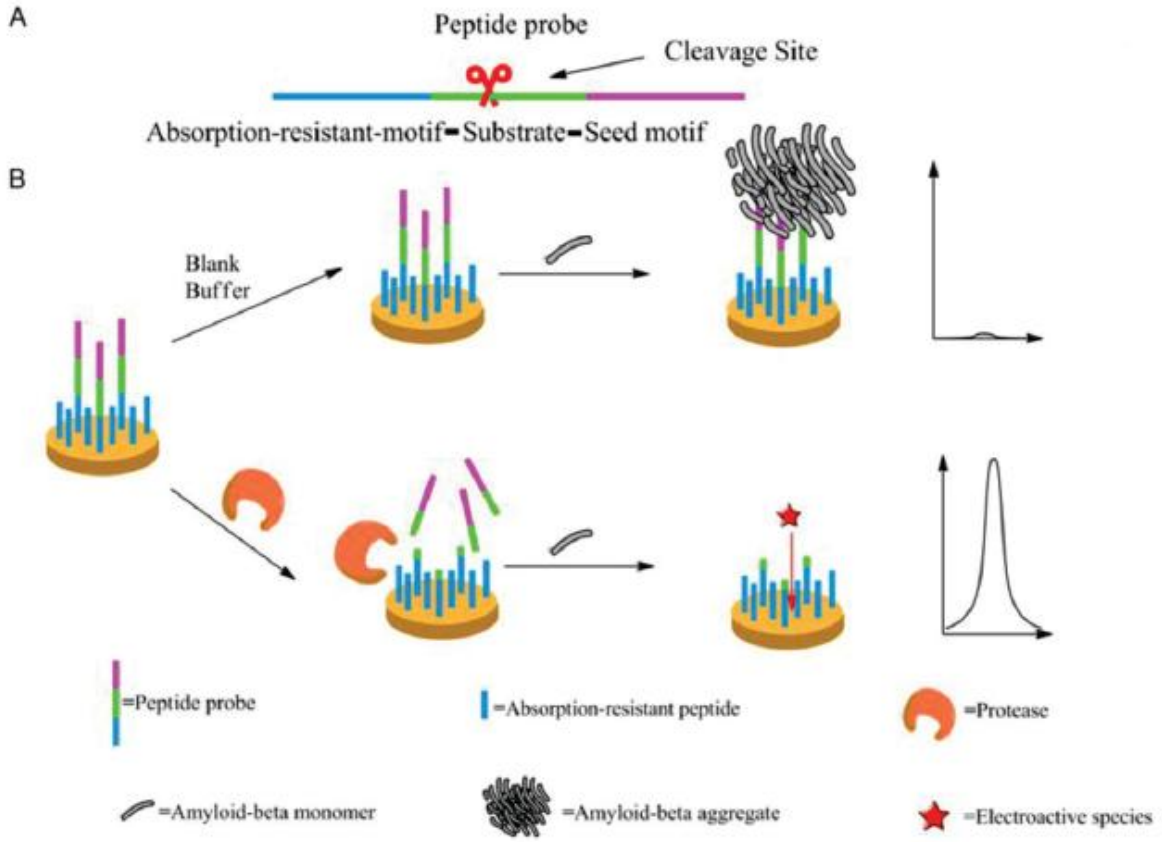

Figure 6. The mechanism of the designed protease assay. (A) The peptide probe showing its motifs. (B) The procedure of the protease assay. Not shown to scale. Reprinted with permission from [28] Copyright 2014 Ivyspring International Publisher.

2.3 Signal amplification

For biomolecule-based electrochemical biosensing, the sensitive and quantitative analysis of ligand-receptor recognition events during signal transduction is a major challenge. Enzyme labels have been extremely useful for the development of sensitive assay strategies because of the opportunity to amplify the generation of signal molecules (the products of enzyme reactions), which are easily converted to signals via electrochemical techniques. Therefore, various strategies involving enzyme reactions have been proposed to enhance the sensitivity of biosensing by the adoption of various amplification systems. Recently, Wu et al. developed an electrochemical biosensor for the detection of protease biomarker from *Bacillus licheniformis* [29]. In the method, a D-amine acid containing substrate peptide with a biotin modifier labeled at its N-terminal (biotin-ALNNd-Ld-LKNNLAC) was

used to capture streptavidin-conjugated alkaline phosphatase (SA-ALP), which catalyzes the conversion of electrochemically inactive 1-naphthyl phosphate into electrochemically active phenol. In the presence of the protease biomarker, the peptide is cleaved, resulting in the remove of biotin moiety from the electrode surface and a decreased electrochemical signal. This method showed a detection limit for protease biomarker detection (0.16 mg mL^{-1}). ALP is one of the most widely used reporter molecules for the amplified electrochemical detection of biomolecules because of its high turnover frequency and high reaction selectivity. The major drawback for this method is the instability of enzymatic product (e.g. p-AP or L-ascorbic acid (AA)) in air. To overcome this defect, reductants can be added to the reaction mixture to protect p-AP or AA from oxidation [30-32]. Moreover, reductants can regenerate p-AP or AA after its electrochemical oxidation at a fast rate, thus enhancing the detection sensitivity. Very recently, Yang's group demonstrated the detection of trypsin based on the proteolytic generation of electroactive 4-amino-1-naphthol by trypsin followed by a signal-amplified electrochemical measurement of 4-amino-1-naphthol using electrochemical–chemical (EC) or electrochemical–chemical–chemical (ECC) redox cycling [33]. 4-Amino-1-naphthol is generated by the cleavage of an amide bond between 4-amino-1-naphthol (denoted as P in the peptide sequence) and oligopeptide (Gly-Pro-Arg) at the C-terminal of Gly-Pro-Arg-P. The results indicated that the detection limits obtained with ECC redox cycling (~1 ng mL^{-1} and ~100 ng mL^{-1} with an incubation period of 2 h and 30 min, respectively) were lower than those obtained with EC redox cycling (~5 ng mL^{-1} and ~200 ng mL^{-1} with an incubation period of 2 h and 30 min, respectively).

AuNPs as labels have been widely used in diagnostics and detection because of their unique characteristics, such as high surface-to-volume ratio, high surface energy, ability to decrease proteins-metal particles distance, and the functioning as electron conducting pathways between prosthetic groups and the electrode surface [34]. Miao et al. have fabricated an electrochemical method for the study of proteolysis using unmodified AuNPs (Fig. 7) [35]. Specifically, substrate peptide of a protease is modified on a gold disk electrode, forming a barrier for electrochemical species and reflecting a significant charge transfer resistance (Rct). After the proteolysis process, the peptide can be cleaved coupled with the decline of Rct. The electrical properties of the substrate residues on the electrode may also change, leading to the subsequent adsorption of AuNPs. Due to the excellent electrical conductivity of AuNPs, Rct can be further decreased, which can be used to reveal the proteolysis process.

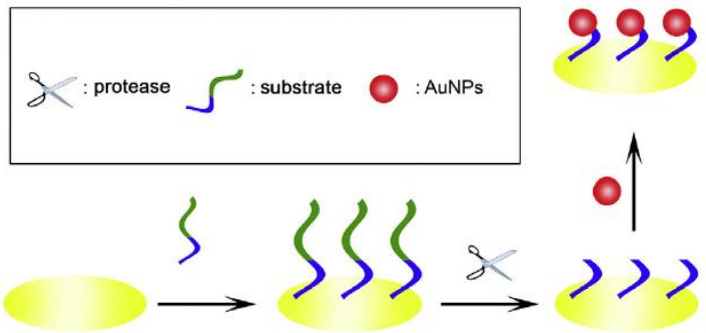

Figure 7. Schematic representation of the electrochemical method for the study of proteolysis. Reprinted with permission from [35]. Copyright 2014 Elsevier.

3. CONCLUSION

Proteases demonstrate high interest and applications in medical and research fields due to their critical role in human physiological reactions, cellular cycles as well as pathogenic agent's life. This work reviewed the progress in the development of electrochemical proteases biosensors. Although there are still limitations for their practical use as regular methods in clinical diagnostic and prognostic, a bright future will arise through the implementation of multiplexed biosensing arrays. Multiplexing can simplify assay procedures, reduce systematic variability and increase predictive accuracy. Moreover, assays that are modular in nature should be designed and carefully implemented for simultaneous detection of multiple biomarkers, as well as to present a platform for high throughput screening prompting rapid disease diagnosis and drug discovery.

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