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Mini review

Recent Developments in Electrochemical, Electrochemiluminescent, Photoelectrochemical Methods for the Detection of Caspase-3 Activity

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Caspase-3 plays an important role in the apoptotic process and has drawn fast growing interests in cancer research, clinical detection and apoptosis-targeted drug discovery. Increasing demands for simple, low-cost and high-performance clinical diagnosis have already promoted the rapid progress in analysis of caspase-3. During plenty of novel methods for caspase-3 determination, electrochemistry-based methods, have been worldly recognized as the most promising technologies because of their simplicity, high sensitivity and specificity. This review aims to briefly summary the recent developments of electrochemical, electrochemiluminescent, photoelectrochemical methods, and provide insights into the incorporation of electroactive molecules or nanomaterials.

Keywords: caspase-3; electrochemistry; electrochemiluminescence; photoelectrochemistry; signal amplification

1. INTRODUCTION

Apoptosis, a highly regulated and complicated process of programed cell death, is critically essential for maintaining normal physiological metabolism and tissue homeostasis [1]. Deregulation of apoptosis program occurs with the development of a variety of severe diseases, including cancer, atherosclerosis, neurodegenerative diseases, autoimmune diseases and so on [2]. Consequently, real time diagnosis of apoptosis at the early stage is of considerable importance to understanding the apoptosis signaling, evaluating the apoptosis-targeted therapy efficiency and monitoring the disease progression. It has been reported that the intracellular apoptosis process is closely related to the activation of a series of cysteine-dependent, aspartate-specific proteases of caspases [3, 4]. In the caspase family, caspase-3 is the most frequently activated cysteine protease and plays a vital role in

both intrinsic and extrinsic apoptotic pathways, which has been recognized as a well-established biomarker of cell apoptosis. Therefore, taking these factors into account, it is of great importance to develop a simple, sensitive and rapid method for highly sensitive detection of caspase-3 activity and screening of its potential inhibitors as apoptosis-mediated potential drugs. With the rapid development of analytical methodologies and instruments, diverse electronic techniques, including electrochemistry (EC), electrochemiluminescence (ECL) and photoelectrochemistry (PEC) have been employed for the detection of bio-related molecules and processes, which have the advantages of rapid, simple, sensitive, and low-cost detection of targets of interest [5]. Therefore, in this present mini-review, we will focus on the EC, ECL and PEC approaches for analysis of caspses-3 based on various signal amplification strategies.

2. EC METHODS

Owing to inherently fast, portable, and cost-effective, EC sensors have proven highly sensitive and selective for biomedical applications. Up to now, a number of EC strategies have been rationally constructed, such as voltammetry (cyclic voltammetry (CV), linear sweep voltammetry (LSV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV), amperometry and electrochemical impedance spectroscopy (EIS) [6]. Yet due to the lack of characteristic electrochemical properties, artificial peptides always need to be tagged with functional molecules or nanomaterials with reversible redox chemistries, convenient redox potentials, as signal markers, which can convert enzyme catalytic cleavage into a readily specific and measurable EC signal. Accompanied with the cleavage of redox-labeled peptide, the efficiency of label transferring electrons to or from the electrode surface also is altered, leading to the change of signal.

The activated caspase-3 can specifically recognize the tetra-peptide sequence of Asp-Glu-Val-Asp (DEVD) on peptide substrate and cleave it after the C-terminal of the motif [3]. Due to the commercial availability in conjugation with various biomolecules, ferrocene (Fc) and its derivatives have been widely employed in EC assays because of their well-defined and reversible redox reaction [7, 8]. In 2008, Li and coworkers first reported a rapid and convenient EC approach to detect apoptosis through monitoring caspase-3 activity by using Fc as an electroactive reporter to label DEVD peptide [3]. As shown in Figure 1, the helix Fc-peptide GDGDEVDGC was specifically cleaved by caspase-3, resulting in the liberating of Fc from the electrode surface and the great signal decline of 85%. This simple method could sensitively detect apoptotic cells without the use of expensive biological instruments and tedious procedures. According to previous reports, p-Nitroaniline (pNA) released from the peptide by hydrolysis reaction showed different redox potential with higher current intensity than pNA-labeled peptide due to the difference in the diffusion constant [9, 10]. Thus, Matsue and coworkers utilized pNA as the electroactive label to detect caspase-3 activity by DPV [11]. As displayed in Figure 2, they successfully evaluated cell apoptosis occurring inside living cells without the requirement of cell lysis, centrifugation and debris removal steps. However, overlap of the reduction of *p*NA and dissolved oxygen in solution may disturb the measurement and the relatively long time for pulse voltammetry further hampers the application for real time monitoring. For this consideration, Matsue's group employed *p*-methoxyaniline (*p*MA)-conjugated peptide for

amperometric detection of caspase-3 activity [12]. Based on this method, caspase-3 was determined with the range of 0.025 – 0.31 units/mL without cell lysis.

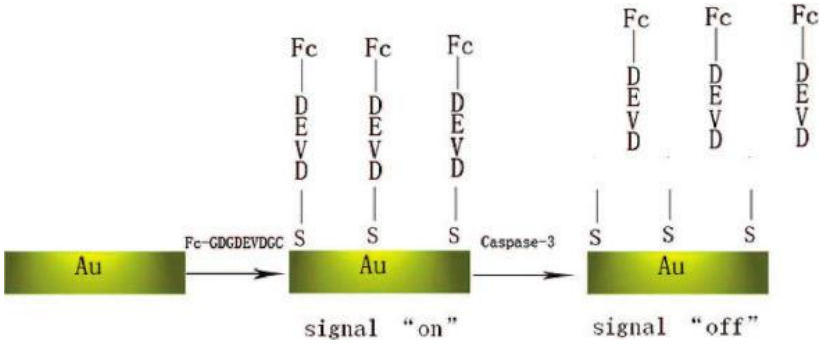

Figure 1. Schematic diagram of electrochemical detection of apoptosis based on Fc-labelled peptide. Reprinted with permission from reference [3]. Copyright 2008 American Chemical Society.

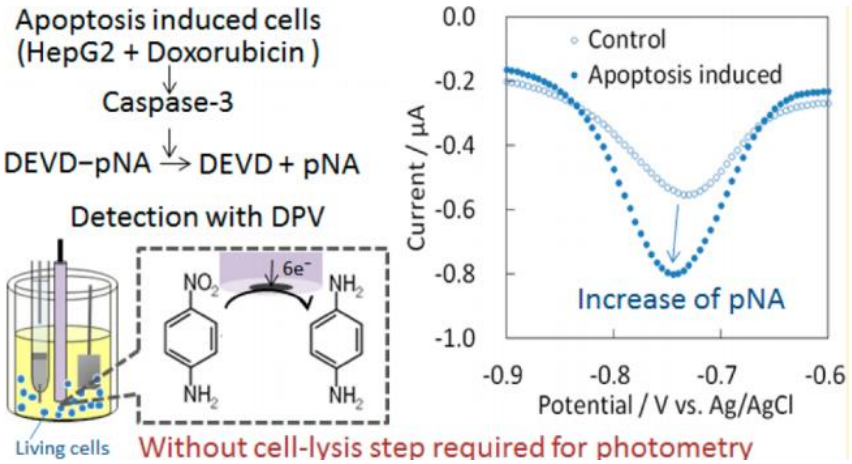

Figure 2. Schematic diagram of electrochemical detection of apoptosis based on *p*NA-labelled peptide. Reprinted with permission from reference [11]. Copyright 2014 American Chemical Society.

To meet the increasing demand of high sensitivity and low detection limit, different signal amplification strategies have been elaborately developed. Among them, enzyme-based signal amplification approach was extensively used, including alkaline phosphatase (ALP) and horseradish peroxidase (HRP) [13]. ALP can catalyze the dephosphorylation process of proteins, nucleic acids, and small molecules, which has been extensively utilized as signal reporter for the development of bio/chem-sensors. For instance, Zhu and coworkers used streptavidin (SA)-labeled ALP to detect caspase-3 activity [14]. In this paper, SA-ALP captured on the surface of the sensor could hydrolyze the substrate 2-phospho-L-ascorbic acid (AAP) into ascorbic acid (AA) which was oxidized on the electrode surface and detected by DPV. Rashidi used SA-coated magnetic beads (MB) to increase the loading efficiency of HRP on the DEVD peptide [15]. HRP, another widely used catalytic enzyme in biosensors, can accelerate the oxidation of the substrate in the presence of H_2O_2. The detection limit of the proposed biosensor was calculated to be as low as 100 pM. To provide the large surface for improving the sensitivity, highly-ordered silica-based mesoporous materials (mobile crystalline

material, MCM-41) were modified on the electrode surface and subsequently decorated with gold nanoparticles (AuNPs) to enhance the electrical conductivity and facilitate the immobilization of DVED peptide [16].

Carbon nanomaterials, such as graphene oxide (GO) and carbon nanotubes (CNTs) are also frequently utilized for signal molecules-loading elements [17]. Moreover, they offer unique advantages including a high surface-to-volume ratio, high electrical conductivity, chemical stability, biocompatibility. For instance, Yin and coworkers designed a "signal on" approach for qualitative and quantitative detection of caspase-3 activities based on GO-assisted electrochemical signal amplification [18]. In this study, casepase-3 catalyzed the hydrolysis of peptide and exposed active amine groups, which could covalently conjugate with graphene oxide through the classical amino coupling chemistry. Next, electroactive methylene blue (MB) molecules were adsorbed on the GO surface through π–π stacking and electrostatic interactions. Finally, this method could sensitively detect caspase-3 in a range of 0.1–100 pg/mL with a low detection limit of 0.06 pg/mL. Nevertheless, the application of coupling reaction during detection procedures and the false positive signal and broad noise background stemming from nonspecific adsorption of highly charged positively or negatively peptide may limit the practical application of this method. Therefore, they used *p*-sulfonatocalix[6]arenes sodium-modified GO (*p*SC_6-rGO) to replace the unmodified GO since calixarene derivatives *p*SC_6 possessed high affinity for the exposed N-terminal amine group and MB through host-guest recognition [19].

In addition to the use of electroactive molecules or enzyme products, some functional nanomaterials are also well acknowledged as electroactive probes for signal amplification, because of the integration of the high-specificity of enzyme cleavage and the excellent physiochemical properties of nanomaterials. For example, after acid dissolution, the metal components of semiconductor quantum dots (QDs) can produce sharp and well-resolved stripping voltammetry signals. Zhu and coworkers developed an EC platform for caspase-3 detection on the basis of QDs functionalized CNTs. The nanoprobe was prepared by the layer-by-layer (LbL) assembly of poly-(dimethyldiallyl ammonium chloride) (PDDA), CNTs and CdTe QDs, followed by coupling with SA through Schiff-bonds. After the cleavage event, the captured nanoprobes of CNTs-QDs-SA were dissolved in HNO_3 for 2 h, and the releasing cadmic ions were quantified by anodic stripping voltammetry (ASV). The signal intensity was dependent upon the amount of intact biotin-DEVD peptide remained on the electrode surface and equally the activity of caspase-3 in cell lysates. Due to their excellent chemical and electrical properties, especially low-redox potential and highly characteristic solid-state Ag/AgCl process, silver nanoparticles (AgNPs) have been extensively employed as electroactive labels to design novel and efficient electrochemical sensing methods [20-24]. Moreover, these methods do not need environmentally hazardous acid dissolution steps. Miao and coworkers reported that AgNPs could be captured the the exposed amino groups of peptide on electrode surface [25]. Then, significant electrochemical signal was recorded by LSV via the Ag/AgCl solid-state process. Chen and coworkers also developed an electrochemical caspase-3 biosensor based on guests involved CB[8] capped AgNPs [26]. Caspase-3 could cleaved the substrate peptide and lead to the exposure of a free phenylalanine at the end. Afterwards, CB[8] specifically bind with the exposed phenylalanine through host-guest

reaction. The AgNPs-based nanostructures were formed through layer-by-layer assembly of AgNPs via electrostatic cross-linking of AgNPs and CB[8], generating an enhanced electrochemical signal.

Recently, the catalytic amplification strategies without the use of extra substrates were favored, for example, methods with H_2O molecule or hydrogen proton as the electrocatalytic substrate [27-30]. Cu(II) complex heterogenized on graphene sheets is reported to exhibit higher catalytic water oxidation efficiency [31]. Liu and coworkers reported that the Cu(II) complexes with amino terminal Cu(II)- and Ni(II)-binding (ATCUN) peptides showed good electrocatalytic ability toward water oxidation at neural pH with lower oxidation potential [32]. Furthermore, they developed a signal-on EC biosensor for evaluation of caspase-3 activity and cell apoptosis by the formation of the Cu(II)-ATCUN metallopeptides on graphene electrode surface as molecular electrocatalysts for water oxidation [33]. In this work, the FFFF sequence-terminated peptide substrate was confined on the graphene electrode surface through the hydrophobic and π-π stacking interactions. After hydrolysis, the ATCUN fragment was exposed, allowing for the formation of the Cu(II)-ATCUN metallopeptides on the electrode surface. A sigmoidal CV wave with an oxidation potential of ~0.82 V was observed in the Cu(II)-containing electrolyte solution. The method achieved a linear range of 0.5 ~ 2000 pg/mL and a detection limit of 0.2 pg/mL

3. ECL METHODS

Due to the high sensitivity and low background, ECL analysis has aroused considerable interest because of the perfect combination of the high-controllable EC assay and sensitive chemiluminescence [34]. In the ECL process, some unstable substances will be produced on the electrode surface, and then they will turn into excited states through the electron transfer reaction, and finally return to the ground state for luminescence. Many efficient ECL systems have been proposed for bioanalysis, such as luminol [35, 36], metal organic complex [37], and nanomaterials [38, 39]. Among those, tris(bipyridine)-ruthenium(II) ($[Ru(bpy)_3]^{2+}$) has been widely utilized in ECL analysis due to its distinguish properties including good water solubility, stability in aqueous solution and high luminous efficiency. Zhu and co-workers first reported an ECL method for the sensitive determination of caspase-3 activity by using $Ru(bpy)_3^{2+}$-doped silica ($Ru@SiO_2$) nanoparticles with tripropylamine (TPA) as coreactant (Figure 3) [40]. In this paper, PDDA-modified CNTs on the GCE were utilized to electrochemically deposite negatively charged AuNPs, which allowed for the modification of biotinylated DEVD peptide through the Au-S interactions. SA-functionalized $Ru@SiO_2$ nanoparticles were captured by the modified electrode via the biotin-SA interaction and then a strong ECL signal was detected in the presence of TPA. After the addition of the apoptotic cell lysates containing active caspase-3, the DVED-containing peptides were specifically recognized and cleaved, resulting in the loss of the biotin label. Meanwhile, the release of $Ru@SiO_2$ nanoparticles on the electrode led to the decrease in the ECL intensity. The proposed method was successfully employed to monitor caspase-3 activity and evaluate anticancer drugs. Besides, Zhou and coworkers demonstrated a novel ECL biosensor for the detection of caspase-3 based on the specific protease cleavage and covalent interaction between cysteine and $Ru(bpy)_3^{2+}$ derivate [41]. After the cleavage, the exposed cysteine in peptide reacted with $Ru(bpy)_3^{2+}$-2-cyanobenzothiazole ($Ru(bpy)_3^{2+}$-CBT) through condensation

reaction under mild conditions. Then, the biotinylated products were enriched and separated by SA-coated magnetic beads (SA-MBs) for subsequent ECL analysis. Moreover, Rashidi and coworkers developed an ECL biosensor for caspase-3 detection based on the widely used luminol/HRP/H_2O_2 system [42]. In this work, rGO-decorated with AuNPs was used to improve the immobilization of DVED peptide and the conductivity of the electrode. Many biotinylated HRP molecules were loaded on the SA-MB as ECL intensity enhancing probes. The biotinylated peptide can capture HRP-SA-MBs and the HRP enzyme enhanced the oxidation and the ECL intensity of luminol in the presence of H_2O_2. Under the optimized conditions, caspase-3 activity was quantified with the linear dynamic range of 0.5-100 fM and the detection limit of 0.5 fM.

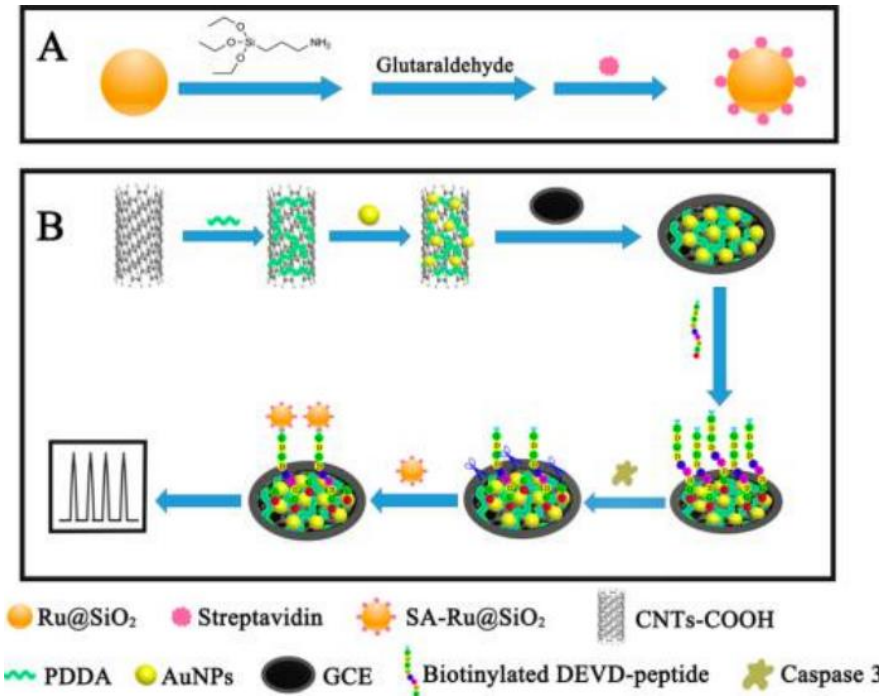

Figure 3. Schematic diagram of the construction of ECL probe (A) and the detection of caspase-3 activity (B). Reprinted with permission from reference [40]. Copyright 2016 American Chemical Society.

4. PEC METHODS

As a new emerging and powerful analytical technique, PEC analysis has aroused growing interest in recent years, due to the successful integration of the PEC process and electrochemical analysis [43, 44]. It possesses advantages of the reduced background noise signal, higher sensitivity and easy miniaturization. In a typical PEC analysis, there are two essential ingredients, PEC active materials and biorecognition elements. Up to now, plenty of photoactive nanomaterials with the narrow band gap and high photon-electron conversion efficiency have been synthesized and applied in construction of novel PEC biosensors, such as semiconductor, metal, carbon materials [45]. Zhu and coworkers proposed a multiple signal amplification PEC strategy for antileukemia drug evaluation (F) (Figure 4) [46]. In this work, photoactive manganese-doped CdS@ZnS core−shell nanoparticles (Mn:CdS@ZnS) were prepared through a simple and novel wet chemical route. When compared with

CdS nanoparticles, Mn:CdS@ZnS nanoparticles could produce high photocurrent signal, which was attributed to the synergistic effect of the metal ion dopant, Mn^{2+} and the protective shell ZnS. Then, the nanoparticles were deposited on the indium tin oxide (ITO) electrode and aminated with poly (ethylene imine) (PEI). Biotin-DVED peptide was utilized to link SA-ALP on the aminated electrode surface through the biotin-SA interaction, respectively. The hydrolysis product of AA is a more efficient electron donor which can generate an enhanced photocurrent signal. However, in the presence of caspase-3, the peptide was cleaved and the amount of ALP bound on the electrode surface was decreased, thus resulting in the weakened photocurrent. The signal is inversely proportional to the amount of caspase-3. Finally, the efficacy of anticancer drug nilotinib was sensitively evaluated, demonstrating the potential of the treatment of chronic myeloid leukemia. Aiming to simplify the testing procedures and eliminate the nonspecific adsorption, Chen and coworkers developed a label-free and blocker-free PEC biosensor for caspase-3 detection [47]. In this work, photoactive nitrogen-doped porous carbon-ZnO nanopolyhedra (NPC-ZnO) was employed to modify the ITO electrode and further was deposited with CdS for the immobilization of the DEVD peptide and the improvement of the photocurrent response of the electrode. The adsorbed peptide blocked the consumption of AA and ensured antifouling of the sensing interface. However, once caspase-3 was added, the DVED peptide was hydrolyzed and removed from the ITO/NPC-ZnO/CdS electrode, resulting in the enhancement of the photocurrent of the electrode. The proposed PEC sensor exhibited a wide linear response range of 0.2~20 ng/mL and a low detection limit of 0.14 ng/mL.

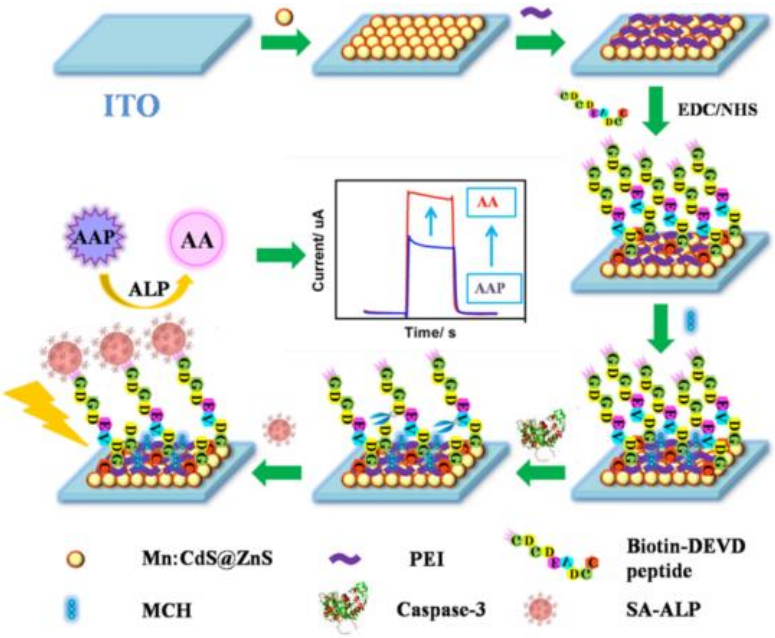

Figure 4. Schematic diagram of fabrication process for the multi-signal amplified photoelectrochemical sensing platform. Reprinted with permission from reference [46]. Copyright 2014 American Chemical Society.

Nanoparticles possessing excellent peroxidase- or catalase-like activity have been used as mimetics to label the substrate peptide as signal reporting probes. For example, a multifunctional signal amplifier for sensitive caspase-3 assay was developed based on p-type semiconducting and

peroxidase-like Co_3O_4−Au polyhedral, which is prepared from zeolitic imidazolate framework (ZIF-67) (Figure 5) [48]. Except the steric hindrance effect, Co_3O_4−Au polyhedral can competitively consume electron donors and exciting light energy, can catalyze the generation of benzo-4-chloro-hexadienone (4-CD) precipitate on the ITO electrode surface. The produced precipitate reduced the photocurrents of the n-type semiconductor Bi_2S_3 and decreased the capture of the photogenerated electrons of Co_3O_4−Au polyhedral. On the basis of this strategy, a wide linear response range of 0.5−50 ng mL^{-1} and a low detection limit of 0.10 ng mL^{-1} for caspase-3 detection were successfully achieved. Besides, gold or silver nanoparticles have also been extensively applied in the development of PEC sensors because they can produce localized surface plasmon resonance (LSPR) under the stimulation of QDs-generated luminescence and the LSPR can in turn modulate the exciton states of CdS QDs. Typically, when the overlap between emission spectrum of QDs and LSPR absorption spectrum of the nanoparticles and the distance between QDs and the nanoparticles is appropriate, gold or silver can quench the photocurrent of QDs through the energy transfer (ET) effect [49, 50]. Based on this principle, Dai and coworkers proposed a PEC approach for apoptosis evaluation by using peptide as the distance controller between oppositely charged AuNPs and CdTe QDs [51]. As illustrated in Figure 6, the designed peptide contained three functional sequences: Au-binding sequence, Cd-binding sequence and DEVD sequence.

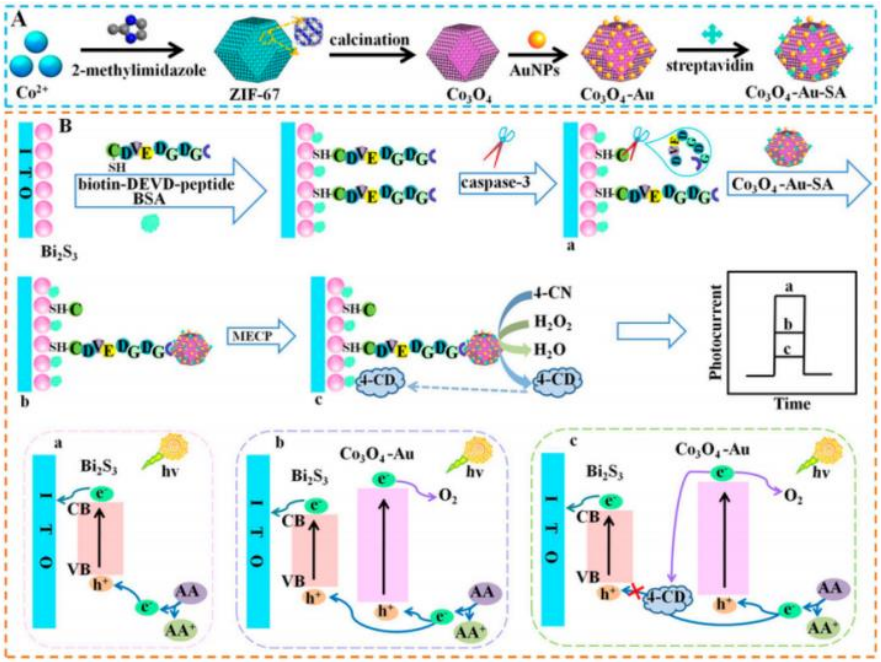

Figure 5. Schematic diagram of (A) preparation procedure of Co_3O_4−Au-SA; (B) Mechanism of a multifunctional p-type semiconducting Co_3O_4−Au polyhedral-based PEC sensor. Reprinted with permission from reference [48]. Copyright 2018 American Chemical Society.

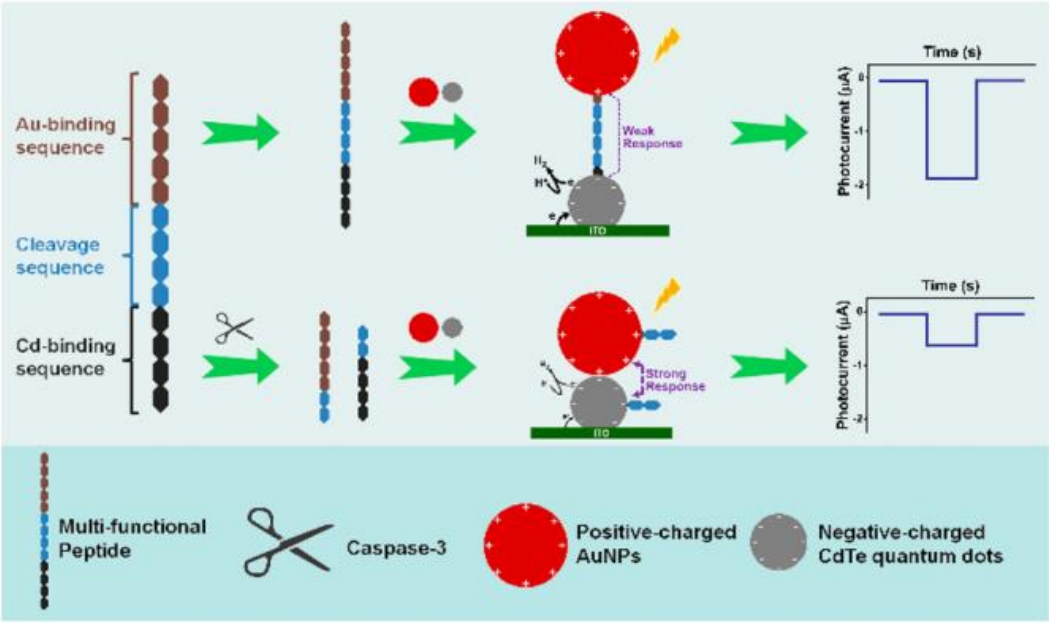

Figure 6. Schematic diagram of principle of caspase-3 detection by peptide- and electrostatic attraction-guided excitonic response. Reprinted with permission from reference [51]. Copyright 2019 American Chemical Society.

Due to the separation of intact peptide and the interaction between the distance sensitive particles, the excitation reaction was weak, and obvious PEC reaction was observed. On the contrary,cleavage of the peptide by caspase-3 induced the aggregation of positive-charged AuNPs and negative-charged CdTe QDs through electrostatic attraction, subsequently enhancing the ET effect and decreasing the PEC response. Caspase-3 was sensitively quantified with a relatively low detection limit (of 58 fg/mL). The method was then used to evaluate staurosporine-induced apoptosis.

Table 1. Analytical performances of the reported methods for caspase-3.

Methods	Signal reporter	Detection limit	Linear range	Refs
DPV	pMA	0-0.001 units/mL	0.002 units/mL	[12]
SWV	HRP	100 pM-1 nM	100 pM	[15]
SWV	HRP	10 fM-10 nM	10 fM	[16]
DPV	GO/eMB	0.1-100 pg/mL	0.06 pg/mL	[18]
DPV	pSC_6-rGO/eMB	10–100 pg/mL	0.0167 pg/mL	[19]
LSV	AgNPs	1–10 ng/mL	24.62 pg/mL	[26]
CV	ATCUN-Cu(II) complex	0.5 pg/mL-2 ng/mL	0.2 pg/mL	[33]
ECL	Ru@SiO_2	0.2–200 pg/mL	0.07 pg/mL	[40]
ECL	$Ru(bpy)_3^{2+}$-CBT	5×10^{-6}-1.5×10^{-3} U/mL	5×10^{-6} U/mL	[41]
ECL	HRP	0.5-100 fM	0.5 fM	[42]
PEC	NPC-ZnO/CdS	0.2-20 ng/mL	0.14 ng/mL	[47]
PEC	Co_3O_4-Au	0.5-50 ng/mL	0.1 ng/mL	[48]
PEC	AuNPs/CdTe QDs	0.1-100 pg/m	58 fg/mL	[51]

5. CONCLUSION

Caspase-3 plays a crucial role in the monitoring of apoptotic process and evaluation of the efficacy of anticancer drugs. Electrochemistry-based biosensors show huge potential and have been widely applied for the detection of caspase-3. Herein, we have reviewed the reported EC, ECL and PEC strategies for caspase-3 detection, especially the signal amplification strategies based on enzymes and nanomateris. For future biosensing development and practical applications, multiplexed and simultaneous sensing of various apoptosis-related biomarkers is of great importance.

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