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Mini review

Electrochemical biosensors for the detection of p53 proteins and anti-p53 autoantibody

Cai-Xia Yu[1,*], *Fan Xiong*[1] *and Lei-Lei Liu*[2,*]

[1]Henan Province of Key Laboratory of New Optoelectronic Functional Materials,College of Chemistry and Chemical Engineering, Anyang Normal University, Anyang, Henan 455000, People's Republic of China
[2]School of Environment and Material Engineering, Yantai University, Yantai 264005, People's Republic of China
[*]E-mail: yucaixiachem@163.com (C. X. Yu), liuleileimail@163.com (L. L. Liu)

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As a tumor suppressor, p53 protein has a deep relationship with the growth of tumor cells and other important cellular processes. The protein has been widely recognized and used as a potential biomarker in clinical diagnoses. Urgent demands for detection of the biomarker have accelerated the emergence of various analytical methods. Electrochemical methods integrated with enzymes or nanomaterials for signal amplification have aroused extensive interest. The aim of this review is to give a brief classification and description of various types of electrochemical biosensors for quantification of wild-type and phosphorylated p53 proteins and anti-p53 autoantibody along with the development trends of the strategies.

Keywords: electrochemical biosensors; p53 proteins; autoantibody; signal amplification

1. INTRODUCTION

p53 protein as the tumor suppressor plays a significant function in cell cycle control and apoptosis. Almost all of its biological roles are dependent largely on the DNA-binding property [1,2]. Over 50% of human cancer cases have been associated with mutations in the p53 gene. Thus, p53 protein is believed to be a reliable molecular biomarker for cancer diagnosis and prognosis [3]. Alongside the mutation, p53 protein can be phosphorylated on the serine and threonine residues within the N- and C-terminal domains by protein kinases [1,2]. Researchers have developed the corresponding antibodies against different phosphorylated residues of p53 protein. However, it is still desirable to develop a rapid, ultrasensitive and selective method for determining the contents of wild-type and phosphorylated p53 proteins. The currently used methods for measuring endogenous wild-

type p53 protein are electrophoretic mobility shift assay (EMSA) and antibody-based enzyme-linked immunosorbant assay (ELISA). The EMSA is simple, but to some extent semiquantitative. ELISA, relying on a sandwich immunoassay, requires multiple steps and the use of labeled antibody and special substrates. Recently, much attention has been devoted to develop novel techniques, such as surface plasmon resonance, electrochemical methods, optical techniques and so on [4,5]. Among them, electrochemical biosensors exhibit great application prospects in view of its inherent advantages, including high sensitivity and specificity, rapid-responsive and compatibility with miniaturization, and relatively low-cost detection [6]. In this review, we provide an overview of various types of electrochemical biosensors for quantification of wild-type and phosphorylated p53 proteins and anti-p53 autoantibody along with the development trends of the strategies.

2. P53 PROTEIN DETECTION

The levels of p53 protein in human serums are low. In order to meet the demand of sensitivity, signal-amplified electrochemical techniques based on nanomaterials have been developed and extensively applied to the highly sensitive detection of p53 protein in the past decades. There are mainly two types of detection modes: electrochemical impedance spectroscopy (EIS)-based label-free detection and sandwich-like detection with enzymes, nanomaterials or electroactive molecules as signal labels.

2.1 EIS methods

Among different electrochemical techniques, EIS gained considerable interest as a bioanalytical method because of its sensitivity and label-free feature. By measuring the change in impedance during the biorecognition event on the electrode surface, the target concentration can be determined [7]. This label-free method has thus been used for sensitive and selective detection of p53 protein [8]. For example, Yeo et al. reported an EIS immunosensor through the interaction between p53 protein and its antibody [9]. In this study, to conserve natural conditions and enhance the affinity to its antibody, the p53 core domain protein with a natural folding structure was used as a mode. (R)-lipo-diaza-18-crown-6 (lipo-diaza crown) was used to form a self-assembled monolayer (SAM) on the gold disk electrode for the immobilization of antibody through host-guest interaction between crown and alkylammonium group on antibody surface. As shown in Figure 1, after immuno-interaction events with increasing concentrations of p53 protein, the blocking effect caused by the protein limited the charge transfer of $[Fe(CN)_6]^{3-/4-}$ to the electrode surface, thus leading to a linear increase in the charge transfer resistance (R_{ct}). On basis of this principle, the simple and label-free method achieved a linear concentration range of 0 ~ 5 μg/mL with a detection limit of ~0.10 μg/mL (~ 4 nM). To improve the performance of biosensor, it is a useful and powerful to modify the electrode with nanomaterials or polymers [10,11]. These interface materials can increase the conductivity of electrode and increase the active surface area for immobilization of biorecognition elements, thus amplifying the electrochemical signal. For example, gold nanoparticles (AuNPs) decorated electrochemically reduced graphene oxide (ERGO) and chitosan/conductive carbon black composite were utilized to modify electrodes for

developing electrochemical immunosensors for the detection of p53 protein and its antibodies [12,13]. Furthermore, to achieve early diagnosis and multiplexed detection, Xie et al. developed a disposable easy-to-use electrochemical microfluidic chip by incorporating six kinds of antibody biomarkers into the specially designed three electrodes system, including anti-p53 antoantibody [14].

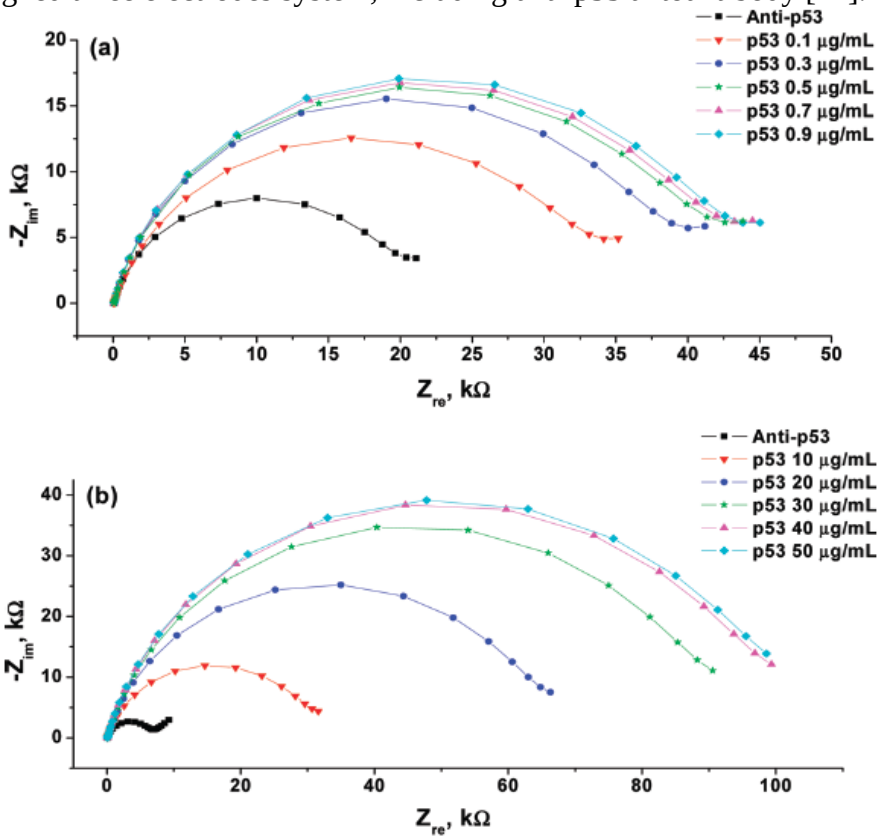

Figure 1. EIS results obtained for immobilized antibodies and antigens in a solution containing a redox probe (2.5 mM $Fe(CN)_6^{3-/4-}$each) and 0.25 M KCl. The p53 concentration range was (a) 0.1-0.9 μg/mL and (b) 10-50 μg/mL, respectively. Reprinted with permission from reference [9]. Copyright 2009 American Chemical Society.

2.2 Sandwich-like electrochemical biosensors

The nanomaterials-based electrochemical techniques have attracted considerable interest in biomolecular analysis due to its intrinstic advantages compared to the other methods [15]. In electrochemical detection, the common quantitative estimation of p53 protein was accomplished by measuring the electrochemical response from the interaction between p53 protein and the tagged electroactive molecules. A measurable electrochemical signal was generally obtained by applying different electrochemical voltammetry techniques, including cyclic voltammetry (CV), linear sweep voltammetry (LSV), differential pulse voltammetry (DPV), square wave voltammetry (SWV), and stripping voltammetry. One of the most popular strategies is the use of nanoparticles as an electroactive reporters such as metal NPs and semiconductors, or as carriers to load a large amount of electroactive molecules to amplify the electrochemical signal. These nanomaterials include AuNPs, magnetic microcarriers, magnetic beads (MBs), graphene oxide (GO), Au nanorods (AuNRs) and carbon nanospheres.

p53 tetramer, tetramerized from four wild-type p53 proteins via their C-termini, can specifically bind to the double-stranded DNA (ds-DNA) consensus sites. However, the mutant p53 protein lose the sequence-specific DNA binding ability [16,17]. Fojta and other groups have developed different electrochemical methods by combining with immunoprecipitation at magnetic beads to study p53 protein-DNA interactions and evaluate the effects of antitumor drug cisplatin on the p53-DNA binding [18-22]. Moreover, the specific and strong binding has facilitated the detection of wild-type p53 protein. For example, Wang et al. first reported a signal-amplified voltammetric method for detection of wild-type p53 protein by using dsDNA consensus as the recognition element and ferrocene (Fc)-capped AuNPs/streptavidin conjugates as the reporting signal (Figure 2) [23]. In this work, the thiolated ss-ODN was immobilized on the gold electrode to produce a SAM mixed with hexanethiol (HT), followed by hybridization with its complementary DNA to form dsDNA consensus. Wild-type p53 was captured by the dsDNA consensus. Then, the five metal-free cysteine residues on the exterior of the p53 proteins were derivatized with N-biotinoyl-N'-[6-maleimidohexanoyl] hydrazide (biotin-Mi). Fc-capped AuNPs/streptavidin conjugates were attached to the complexes near the electrode surface via the specific biotin-streptavidin interaction, undergoing a facile and reversible electron-transfer reaction between Fc and electrode. A relatively low detection limit of 2.2 pM was achieved, which is attributed to the attachment of AuNPs capped with 127 ± 10 Fc tags per p53/dsDNA complex [24]. Silver nanoparticles (AgNPs) exhibit a sharp oxidation peak and low-redox potential in aqueous solution, which make them more excellent than AuNPs in electrochemical biosensors. The AgNPs network architecture can amplify the electrochemical signal based on the solid-state Ag/AgCl reaction from AgNPs aggregates to AgCl. Liu's group have reported the detection of microRNAs, glycoproteins and enzymes based on the in situ formation of AgNPs aggregates for signal amplification [25-30]. Recently, the group reported the detection of wild-type p53 protein based on the Ag-S interactions between p53 protein and AgNPs and the signal amplification of benzene-1,4-dithiol (BDT)-triggered in situ assembly of AgNPs on electrode surface [31,32]. The target at the concentration as low as 0.1 pM was readily determined.

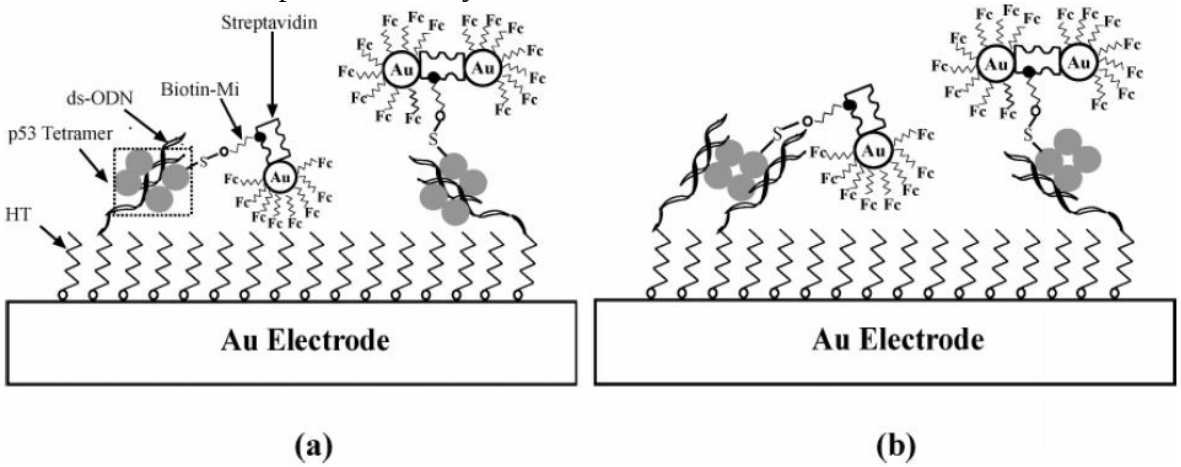

Figure 2. Schematic representations of the capture of p53 by ds-ODN-modified electrodes and the following amplified voltammetric detection of p53 via oxidation of the ferrocene (Fc) tags on the gold nanoparticle/streptavidin conjugates. Reprinted with permission from reference [23]. Copyright 2008 American Chemical Society.

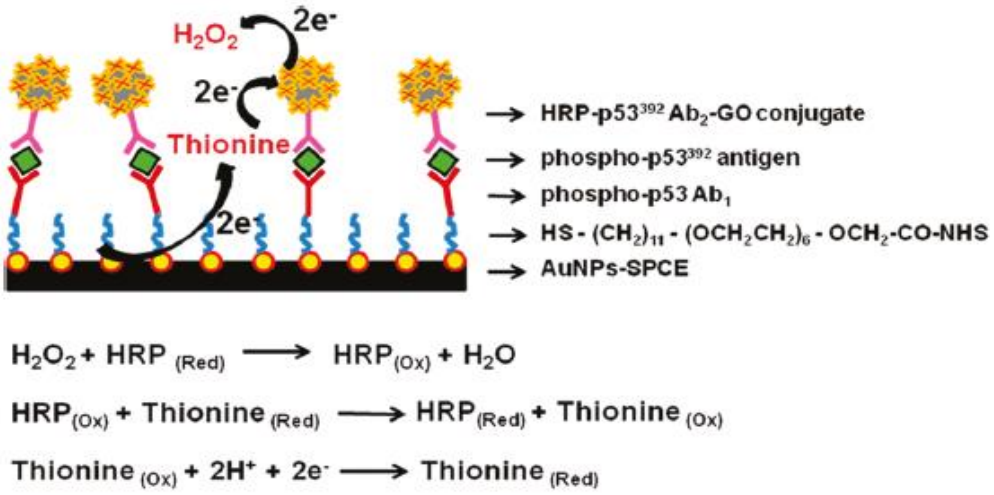

Figure 3. Schematic illustration of the multienzyme labeling amplification strategy using HRP-p53^{392} Ab_2-GO conjugate. Reprinted with permission from reference [33]. Copyright 2011 American Chemical Society.

Electrode substrate is an important element in development of electrochemical biosensors. Many research papers have proved the possibility of improving sensitivity of electrochemical biosensors by tailoring the morphology of nanostructures. Hasanzadeh et al. developed a label-free electrochemical immunosensor for p53 protein detection by using electrodeposited AuNPs and poly-cysteine to modify the electrode surface. The interface provided a higher surface to immobilize large numbers of antibody molecules and accelerating the rate of electron transfer [11]. Enzyme amplification is a desirable and effective choice for development of electrochemical biosensors because one enzyme molecule can catalyze the conversion of tens of thousands of substrate molecules per minute. Horseradish peroxidase (HRP) has been used to label secondary anti-p53 Ab_2 against phosphorylated p53 on Ser15 (phospho-p53^{15}). The enzyme catalyzed oxidation of thionine in the presence of hydrogen peroxide (H_2O_2) which can be electrocatalytically reduced [34-36]. In a competitive amperometric immunosensor reported by Giannetto et al., alkaline phosphatase (ALP)-conjugated antibody was also employed to detect immunosorbed anti-p53 antibodies for indirect quantification of the level of p53, in which ALP dephosphorylated non-electroactive hydroquinone diphosphate into electroactive hydroquinone (HQ) [37]. However, the low ratio of enzyme to antibody (1:1) limited the sensitivity of biosensors. To achieve signal amplification, NPs were used to load a large amount of enzymes for an individual sandwich immunological reaction event. For example, Lin's group explored the GO as a nanocarrier in the controlled loading of HRP and phosphop53^{392} signal antibody (p53^{392}Ab_2) for ultrasensitive phosphorylated p53-S^{392} (phospho-p53^{392}) detection (Figure 3) [33]. In this work, GO with high-density carboxyl functional groups facilitated the co-immobilization of HRP and p53^{392}Ab_2 at a high ratio. After a classical sandwich immunoreaction, the HRP-p53^{392}Ab_2-GO captured onto the electrode surface produced an multienzyme amplification electrocatalytic response by the reduction of enzymatically oxidized thionine in the presence of hydrogen peroxide. The signal response was gained by CV and SWV techniques. The proposed immunosensor showed excellent performance for accurate quantification of phospho-p53^{392} with a wide linear range from

0.02 to 2 nM. The detection limit of 0.01 nM was achieved.

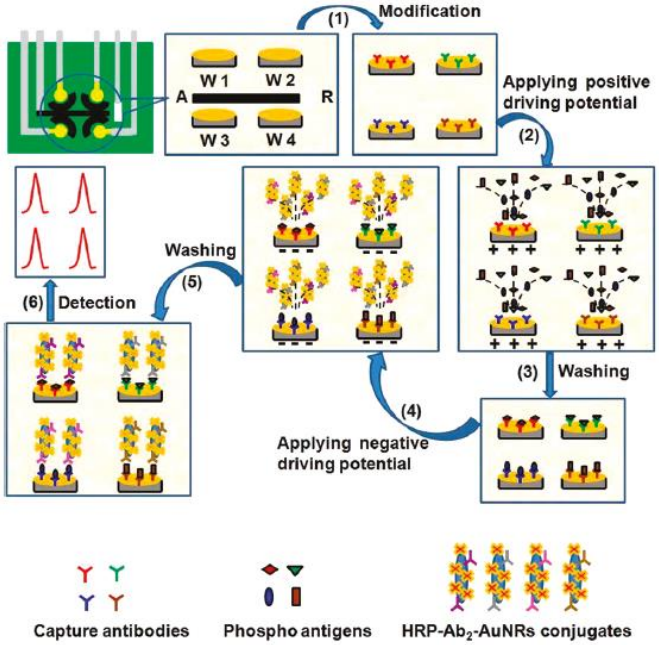

Figure 4. Schematic illustration of multiplexed electrochemical immunoassay by an electric field-driven process and multienzymes labeling amplification strategy using HRP-Ab_2-AuNRs conjugates. Reprinted with permission from reference [38]. Copyright 2011 American Chemical Society.

Moreover, Lin's group combined enzyme amplification with electric field-driven strategy to develop multiplexed electrochemical immunoassay for fast and sensitive detection of phospho-$p53^{392}$, phospho-$p53^{15}$, Ser46 (phospho-$p53^{46}$), and total p53 simultaneously (Figure 4) [38]. In this paper, AuNRs were applied to load HRP and Ab_2 to improve the sensitivity. During the simultaneous immunoassay procedure, the immunosensor was applied with 0.4 V for 3 min and then -0.2 V for 1.5 min to accelerate the immunoreaction processes through the electric field-driven method. In this method, the positive driving potential facilitated the transport of negatively charged antigens to the electrode surface and a low negative driving potential help positively charged HRP-Ab_2-AuNRs to form sandwich immunocomplexes on each working electrode. Under the optimal conditions, this method with greatly amplified sensitivity and shortened time had a wider linear range and a lower detection limit than that of ELISA. Owing to their intrinsic advantages of good stability, versatility in chemical modification, and ease of separation, magnetic beads (MBs) were also utilized to provide a large surface to load capture antibody and facilitate the separation of the immunocomplex from biological samples under magnetic field [39]. Luo's group proposed a magnetic electrochemical immunosensor for the detection of phospho-$p53^{392}$ on the basis of HRP and Ab_2-loaded carbon nanospheres (CNs) and antibody-modified MBs [40].

Although enzymatic biosensors amplify the signal with high selectivity, they still suffer from instability, critical microenvironmental factors, dependence of dissolved O_2 and/or introduction of a mediator. Thus, it is necessary to propose enzyme-free biosensors for targeted biomolecules. Alternatively, nanomaterials containing tens of thousands of metal ions can be used as effective electroactive probes for the construction of electrochemical biosensors, such as semiconductor

quantum dots and AgNPs. For example, apoferritin, a native nanostructured protein, was used as the template to synthesize compositionally encoded metallic phosphate NPs as tags, which could release the metal components from the NPs tags at pH 4.6 buffer solution and was quantitatively determined by SWV [41]. Chen et al. employed protein cage nanoparticles to template lead phosphates, which was further linked to the $p53^{15}$ detection antibody ($p53^{15}$ Ab_2)-modified CNs as a label [42]. They also developed an electrochemical immunosensor for simultaneous detection of $phosphop53^{15}$ and $phospho\text{-}p53^{392}$ by using SiO_2@Au nanocomposites to carry apoferritin templated cadmium phosphates and lead phosphates as signal reporters, respectively.[27] Recently, mesoporous or hollow NPs are used as nanocontainers for the entrapment of enzymes and small molecules for the signal amplification in various biosensors, including electrochemical, photoelectrochemical, colorimetric and fluorescence methods [43-45]. The attractiveness of easy-to-operate and portable electrochemical biosensors stimulate the development of novel biosensors based on certain commercially available analysis instruments, such as a commercial personal glucose meter, pH meter and barometer. Zhao et al. encapsulated tens of thousands of glucose molecules (about 4×10^5) into antibody-tagged liposome as an amplifier and used glucose meter to detect glucose molecules released for indirectly quantifying phosphorylated p53 protein ($phospho\text{-}p53^{15}$) [46].

Ongoing efforts in research of enzyme mimics and nanotechnology have realized the application of many nanomaterials with catalysis ability as artificial enzyme-like labels for signal amplification [47]. Prussian blue nanoparticles (PB NPs) with high peroxidase-mimetic activity have been extensively used in electrochemical sensors. In an enzyme-free electrochemical immunoassay, Liu et al. prepared gold-PB hybrid microspheres (GPMS) via the reverse micelle method for labeling of anti-p53 Ab_2 for sensitive detection of p53 protein [48]. mAb_1 was immobilized on the screen-printed carbon electrode (SPCE) through a typical carbodiimide coupling. pAb_2 was conjugated onto the GPMS by the strong interaction between cysteine or NH^{3+}-lysine residues of antibody and gold nanoparticles. PB NPs in the hybrid nanocomposites catalyzed the reduction of H_2O_2 and acted as the electron mediators, resulting in the amplification of the electrochemical signal. This designed method exhibited a linear range of 0.5 to 80 U/mL and a detection limit of 0.1 U/mL, which are comparable to those achieved by commercialized ELISA kits.

3. DETECTION OF P53-SPECIFIC AUTOANTIBODY

Autoantibody against the tumor-related antigen can serve as the indicator of tumorigenesis, metastasis and therapy. The anti-p53 autoantibody in serum has been found in 10 ~ 40% of patients dependent upon the type of tumors. Recently, several groups have developed electrochemical biosensors for the detection of anti-p53 autoantibody in view of their simplicity and versatility. For example, María et al. developed the first electrochemical disposable biosensor by conjugating MBs with HaloTag fusion p53 protein (HaloTag-MBs) for the selective capture of p53-specific autoantibody [49]. As depicted in Figure 5, p53-specific autoantibody captured by the HaloTag-MBs was detected with a secondary HRP conjugated antihuman IgG. With the aid of the magnetic separator, the MBs bearing the immunocomplexes were magnetically concentrated on the SPCE. Electrochemical reduction of the products from the HRP-enzymatic oxidation of hydroquinone (HQ) by H_2O_2 generated

the amperometric signal. The signal was depended on the levels of anti-p53 autoantibody in the sample. Under the optimized experimental conditions, the autoantibody was readily determined in the range of 1.1-5 U/mL and the LOD was calculated to be 0.34 U/mL.

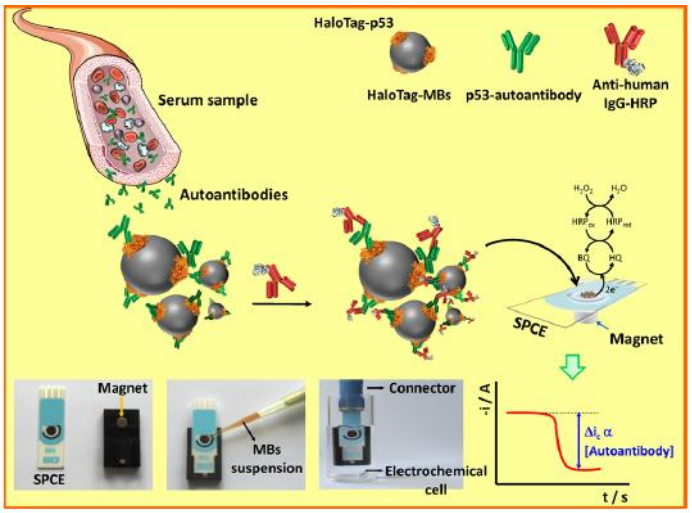

Figure 5. Scheme of the HaloTag fusion protein modified MB-based immunosensing platform for the amperometric determination of p53-specific autoantibody. Reprinted with permission from reference [49]. Copyright 2016 American Chemical Society.

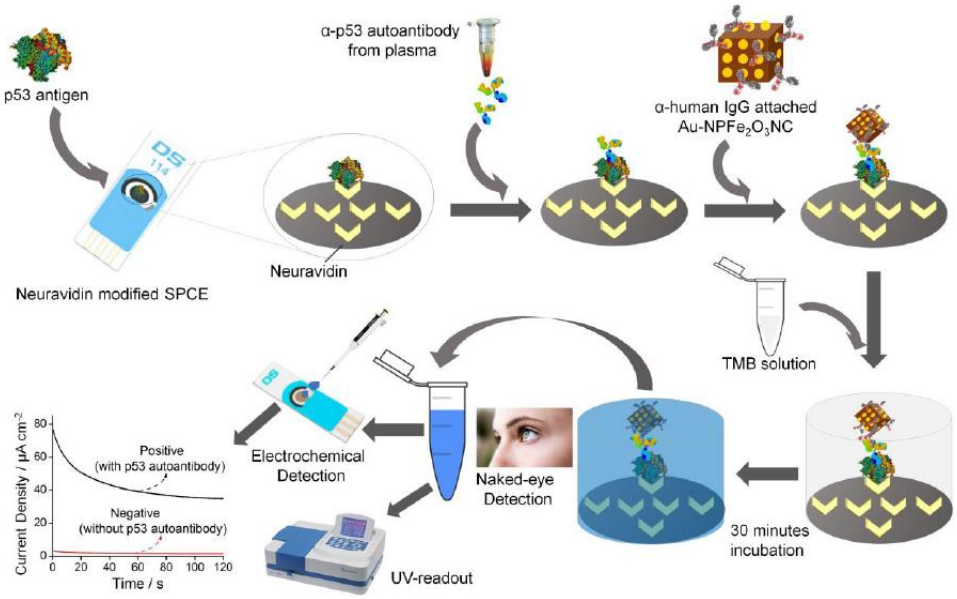

Figure 6. Schematic representation of the assay for the detection of tumor-associated plasma (and serum) p53 auntoantibody. Reprinted with permission from reference [50]. Copyright 2017 American Chemical Society.

The unique magnetic characteristics of magnetic macro/nanoparticlesfor easy separation by magnetic fields facilitate the application of the nanomaterials in bioassays. Yadav et al. reported a spectrophotometric and electrochemical method for anti-p53 autoantibody in which gold-loaded

superparamagnetic nanoporous ferric oxide nanocubes (Au−NPFe_2O_3NC) derived from PB nanocubes were synthesized [51]. Moreover, magnetic nanoparticles and their composite nanomaterials have other intrinsic properties, such as intrinsic enzyme-mimicking activity to catalyze redox reactions of various organic and inorganic compounds. For this view, Mostafa et al. reported the electrocatalytic and colorimetric (naked eye) detection of anti-p53 autoantibody in serum or plasma samples with Au−NPFe_2O_3NC as the signal label (Figure 6) [50]. In this work, Au−NPFe_2O_3NC was used as the nanozyme with enhanced HRP-like activity at room temperature and as the electrocatalyst with improved catalytic activity toward the electroactive molecules [i.e., hexaammineruthenium(III) chloride, ($Ru(NH_3)_6Cl_3$)] [52]. Biotinylated p53 antigen was modified onto the neuravidin-modified screen-printed carbon electrode to selectively recognize and capture the anti-p53 autoantibody through conventional biotin-avidin chemistry. After the target anti-p53 autoantibody was captured to form the immunocomplex with IgG/Au−NPFe_2O_3NC nanocatalysts, an amperometric current was obtained with the freshly prepared (3,3',5,5'-tertamethylbenzidine) TMB/H_2O_2 solution. The stable yellow-colored product from the oxidation of TMB was electroactive. The detection limits were found to be 0.12 U/mL for the colorimetric readout and 0.08 U/mL for the amperometric readout, which are lower than that of Halotag fusion protein-based electrochemical platform (0.34 U/mL).

4. CONCLUSION

p53 protein plays a crucial role in cellular proliferation, tumor suppression, gene regulation and controlling cell growth by activating apoptosis of damaged cells. Various electrochemical strategies to detect wild-type and phosphorylated p53 proteins and their autoantibody by employing enzymes or functional namomatrials as labels have already been reviewed. Although extensive efforts have been conducted to improve the efficacy of biosensors in various sensing methodology, there is still much room to design more perfect electrochemical biosensors for the point-of-care diagnostics. This review will be helpful for the design of novel electrochemical biosensors for the detection of p53 proteins and anti-p53 autoantibody.

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