Chapter 12
Biosensors Based on Supersandwich Assays

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Abstract Only one signal probe is usually bound to the target in traditional sandwich assays, which limits the detection sensitivity. To overcome this limitation, supersandwich assays amplifying the signal through integration of multiple signal probes together have been developed in recent years. In this chapter, we highlight biosensors based on supersandwich assays for the detection of proteins, nucleic acids, small molecules, ions, and cells by a series of efforts reported in the past decade. The detection technologies employed in design of biosensors based on supersandwich assays contain electrochemical assay, electrochemiluminescence assay, fluorescence assay, and surface plasmon resonance assay.

Keywords Supersandwich assays · Multiple signal probes · Electrochemical assay Electrochemiluminescence assay · Fluorescence assay · Surface plasmon resonance assay

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12.1 Introduction

The sandwich assays have achieved great success in detecting proteins, nucleic acids, small molecules, ions, and cells [1]. Usually, only one signal probe specifically hybridizes with a target in a traditional sandwich assay. Therefore, traditional sandwich assays show relatively low sensitivity because the total signal gain is limited. To overcome this limitation, some approaches combining multiple signal probes together in a sandwich assay to amplify the detection signal have been developed as a kind of sandwich assays, namely, supersandwich assays.

The early and classic example of a supersandwich assay was pioneered by Xia, Zuo, Plaxco, and Heeger in 2010, as shown in Fig. 12.1 [2]. Aiming at the limitation that a target hybridizes with a signal probe in a traditional sandwich assay, they innovatively made a modified signal probe that contained a methylene blue (a redox moiety) label and a "sticky end." The target is hybridized with the signal probe, and the sticky end remained free, which can hybridize with another target.

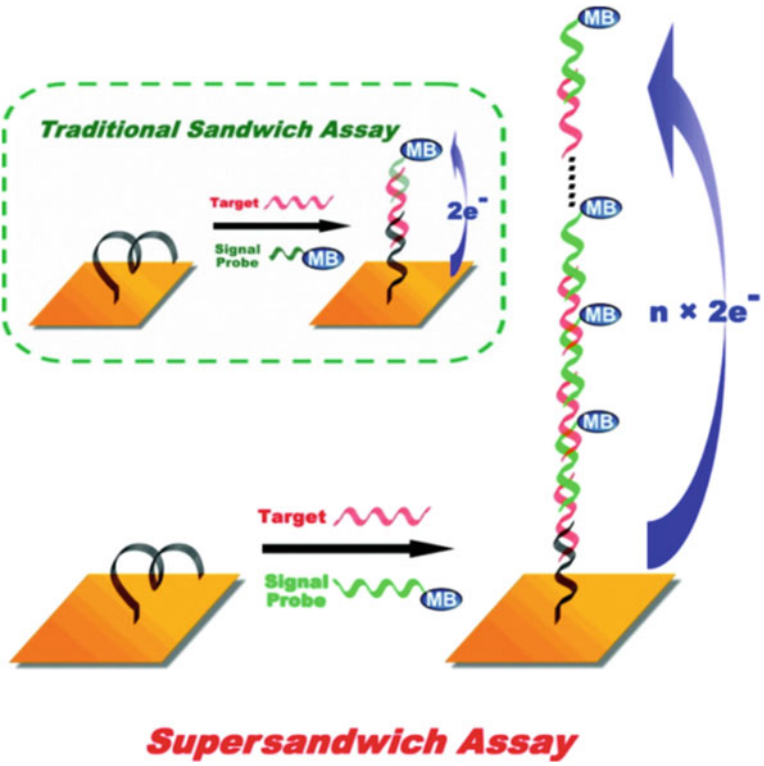

Fig. 12.1 Classic example of a supersandwich assay in which the signal probes hybridize to both ends of the target probe to generate long concatamers, which possess multiple target molecules and signal probes. Inset is the scheme of the traditional sandwich assay. (Reprinted with the permission from Ref. [2]. Copyright 2010 American Chemical Society)

Finally, a supersandwich DNA structure with multiple labels was created. This approach led to a significant improvement in detection limit, compared to a traditional sandwich assay. The former had a detection limit of 100 fM, which the latter had a detection limit of 100 pM.

After that, supersandwich assays have been booming in development [3]. In addition to traditional sandwich assays, supersandwich assays have also been widely used in the detection of proteins, nucleic acids, small molecules, ions, and cells. Similarly, electrochemical assay, electrochemiluminescence assay, fluorescence assay, and surface plasmon resonance assay have still been employed in biosensors based on supersandwich assays. According to the detection objects, we divide the assays into four categories: protein detection, nucleic acids detection, small-molecule and ion detection, and cell detection. In each category, the highlighted examples are classified on the basis of the detection technologies.

12.2 Supersandwich Assays for Protein Detection

12.2.1 Electrochemical Supersandwich Assays

In 2011, Wang et al. proposed an electrochemical immunosensors based on supersandwich multienzyme-DNA label for the detection of Interleukin-6 (IL-6) as a model protein, a biomarker for several types of cancer [4]. The sequence 1 (S1) was conjugated to the secondary antibodies (anti-IL-6) through binding streptavidin of S1 to the biotin tag of anti-IL-6. Then, the capture probe S1 was hybridized with the signal probe S2 with horseradish peroxidase (HRP), which was further hybridized with the target DNA S3, to afford supersandwich multienzyme-DNA label. Supersandwich DNA structure significantly enhanced the amperometric signal, thus achieving a detection limit of 0.05 pg mL^{-1} relative to that of 5.0 pg mL^{-1} using the traditional sandwich label. They then designed an electrochemical biosensor for the detection of folate receptor based on the protecting effect of folate receptor toward folic acid-modified DNA and the signal amplification of supersandwich DNA structure to achieve a detection limit of 0.3 ng mL^{-1}, which approached clinically relevant concentrations of folate receptor [5]. They also described an electrochemical biosensor for the detection of thrombin with a detection limit of 10 pM based on G-quadruplex-linked supersandwich structure [6]. Wang et al. used the aptamer with the high affinity to fabricate a label-free supersandwich electrochemical biosensor for the detection of myoglobin, one of the early biomarkers to increase after acute myocardial infarction, based on target-induced aptamer displacement with a detection limit of 10 pM, which was lower than that of those previous antibody-based biosensors for the detection of myoglobin [7].

The high affinity of the negative phosphate backbone of DNA to positively charged metal cations provides an approach to construct metal nanoclusters/nanoparticles along with the DNA template [8]. The metal nanoclusters/nanoparticles possess mimics' enzyme activity, thus having been paid more and

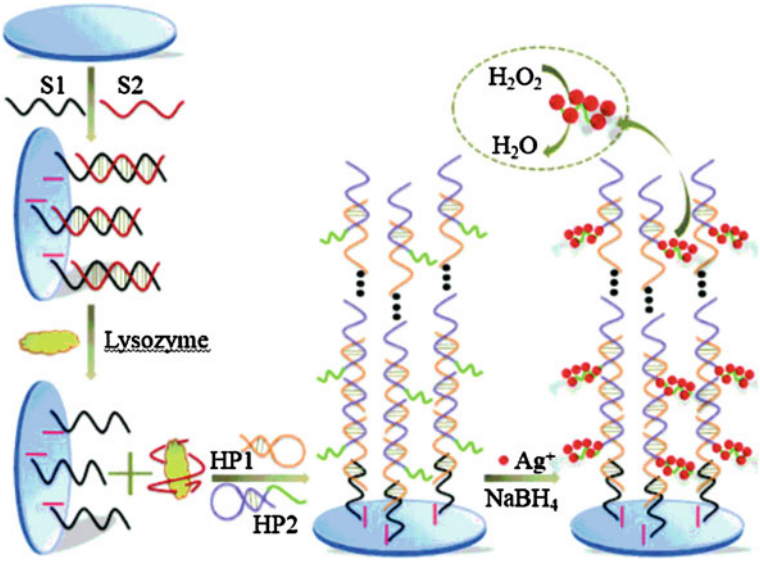

Fig. 12.2 Schematic illustration of an electrochemical supersandwich assay for the detection of protein lysozyme. DNA S1 and DNA S2 are assembled on the gold electrode. S2 is removed by lysozyme and the left S1 triggered the further HCR process. In the presence of Ag^+ and $NaBH_4$, DNA/AgNCs are yielded on the supersandwich DNA structure. Based on the peroxidase-like character of DNA/AgNCs, the lysozyme could be detected. (Reprinted with the permission from Ref. [10]. Copyright 2015 The Royal Society of Chemistry)

more attention in recent years [9]. Wang et al. fabricated an amplified electrochemical aptasensor for the detection of lysozyme based on the mimic oxidase catalytic character of DNA-stabilized silver nanoclusters and hybridization chain reactions (HCR) for signal amplification, as shown in Fig. 12.2 [10]. The DNA duplex was anchored onto the gold electrode and then S2 was specially bound by lysozyme. The left S1 on the surface of the gold electrode triggered HCR of HP1 and HP2 to generate supersandwich DNA structure. Ag^+ attached to the cytosine-rich sequence on the 3'-end of HP2 was reduced by $NaBH_4$ to generate DNA/Ag nanoclusters, which had the peroxidase-like character for the detection of lysozyme with a detection limit of 42 pM. Recently, a supersandwich electrochemical immunoassay based on in situ DNA template-synthesized Pd nanoparticles as signal label was proposed through hybridization proximity-regulated catalytic DNA hairpin assembly strategy for the detection of carcinoembryonic antigen with a detection limit of 0.52×10^{-16} g mL^{-1} [11].

12.2.2 Electrochemiluminescence Supersandwich Assays

DNA methylation plays a significant role in the epigenetic regulation of genomic imprinting, X chromosome inactivation, aging, and carcinogenesis [12]. DNA

methylation has become a potential tumor biomarker for a variety of diseases [13]. DNA methylation generally occurs at cytosines in CpG dinucleotides in the mammalian genome along with the catalysis of DNA methyltransferases (MTase) [14]. Therefore, the detection of MTase activity is of significant importance for early cancer diagnosis [15, 16]. Li et al. developed a label-free supersandwich electrochemiluminescence (ECL) assay for the detection of DNA methylation and the methyltransferase activity with a detection limit of 3×10^{-6} U mL^{-1} [17]. The cytosine residues of supersandwich DNA structure immobilized on the surface of the gold electrode were methylated through introducing M. SssI and S-adenosylmethionine. Using *Hpa*II endonuclease cleaved the un-methylated cytosines, causing the decrease of ECL signal that was derived from $Ru(phen)_3^{2+}$ (an ECL reagent) intercalated into the grooves of dsDNA. Recently, they reported an ultrasensitive ECL biosensor for the detection of DNA demethylase activity through combining MoS_2 nanocomposite with supersandwich DNA structure [18]. A label-free, sensitive, and signal-on ECL assay for the detection of MTase activity with a detection limit of 6.4×10^{-3} U mL^{-1} was developed [19]. The methylation of the dsDNA probes on the sensing electrode inactivated the restriction enzyme activity and inhibited subsequent HCR, resulting in the recovery of the ECL signal of the oxygen/persulfate ($O_2/S_2O_8^{2-}$) system.

$Ru(phen)_3^{2+}$ and its derivatives are well-known ECL luminophores that could be intercalated into the grooves of dsDNA [20]. Yuan et al. fabricated a supersandwich ECL assay for the detection of thrombin with a detection limit of 1.6 fM based on $Ru(phen)_3^{2+}$-functionalized hollow gold nanoparticles as signal-amplifying tags [21]. They further employed histidine as a co-reactant of $Ru(bpy)_3^{2+}$ to amplify ECL signal to fabricate a supersandwich ECL assay for the detection of carcinoembryonic antigen with a detection limit of 33.3 fg mL^{-1} [22]. They also developed a supersandwich ECL assay based on mimic-intramolecular interaction for the detection of prostate-specific antigen (PSA) with a detection limit of 4.2 fg mL^{-1}, as shown in Fig. 12.3 [23]. MWCNTs@PDA-AuNPs bound capture antibody (Ab_1). The PAMAM dendrimer conjugated Ab_2 and supersandwich DNA structure. The detection antibody PSA was immobilized between Ab_1 and Ab_2. The ECL luminophore $Ru(dcbpy)_3^{2+}$ and co-reactant (histidine) were integrated into supersandwich DNA structure to amplify the ECL signal.

12.3 Supersandwich Assays for Nucleic Acid Detection

12.3.1 Electrochemical Supersandwich Assays

The creative case of supersandwich electrochemical assay for nucleic acid detection was reported by Xia et al. [2] as described in the introduction. Inspired by their work, numerous efforts focusing on the electrochemical biosensors for the detection of nucleic acid based on supersandwich assays have been employed so far [24–31]. As an example, an electrochemical biosensor was developed for the detection of

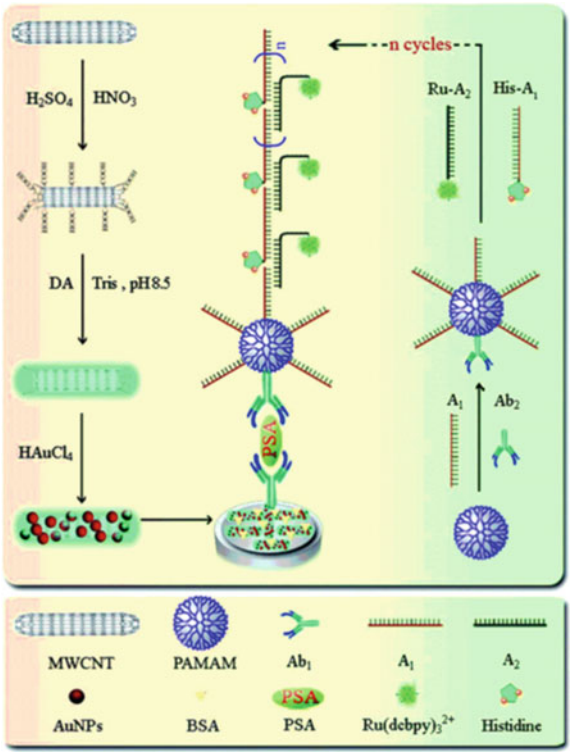

Fig. 12.3 Schematic illustration of an ECL supersandwich assay for the detection of protein PSA. The glassy carbon electrode is modified by MWCNTs@PDA-AuNPs. A PAMAM dendrimer is used to immobilize the detection antibody and supersandwich DNA structure. The supersandwich DNA structure containing multiple Ru $(dcbpy)_3^{2+}$ and histidine further amplifies the ECL signal. (Reprinted with the permission from Ref. [23]. Copyright 2014 The Royal Society of Chemistry)

microRNA (miRNA) based on a catalytic hairpin assembly and supersandwich amplification [31]. The target miRNA-221 (a potentially useful biomarker of cancers) triggered the assembly of molecular beacons H1 and H2 to form H1–H2 complexes followed by releasing miRNA-221. H1–H2 complexes were captured on the electrode and further hybridized with HRP-DNAs as signal tags to produce supersandwich DNA structure on the electrode. The reaction of 3,3′, 5,5′-tetramethylbenzidine (TMB)/H_2O_2 was catalyzed by HRP to generate amperometric signals that were corresponding to the target miRNA-221. The isothermal dual-amplification strategies without nanoparticles provided high sensitivity and selectivity during detection.

An interesting example that supersandwich DNA structure was constructed on the nanochannel walls to fabricate supersandwich electrochemical assay for the detection of DNA was reported by Xia et al. in 2013, as shown in Fig. 12.4 [32]. The capture DNA probe was first immobilized onto the nanopores and captured the target DNA through hybridization. The signal probes (S1 and S2) were hybridized to create long concatamers, supersandwich DNA structure in the nanopores, which efficiently blocked the pathway for ion conduction. This assay achieved a detection limit of 10 fM for oligonucleotides.

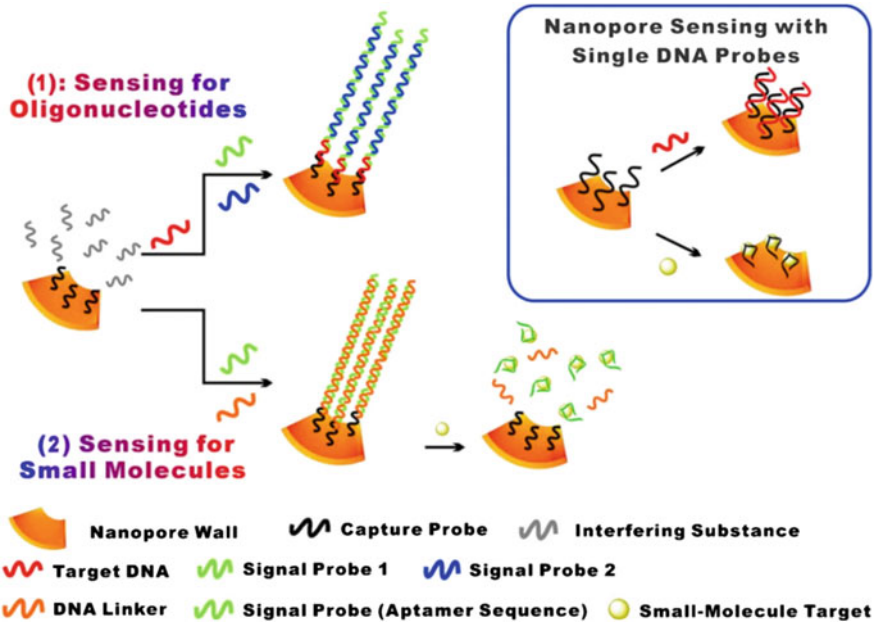

Fig. 12.4 Schematic illustration of an electrochemical supersandwich assay for the detection of DNA. Inset is the scheme of the traditional sandwich assay in which a single capture DNA hybridizes to a single target strand or binds to a single molecular target. The supersandwich electrochemical assay integrates a more complex DNA nanostructure within the nanopores. (Reprinted with the permission from Ref. [32]. Copyright 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim)

12.3.2 Electrochemiluminescence Supersandwich Assays

Zhang et al. described a highly sensitive supersandwich ECL assay for the detection of the human immunodeficiency virus-1 (HIV-1) gene, as shown in Fig. 12.5 [33]. The capture probe was first anchored on the surface of the gold electrode and hybridized with the target HIV-1 gene. Two auxiliary probes were hybridized with the target HIV-1 gene to generate supersandwich DNA structure on the surface of the electrode. $Ru(phen)_3^{2+}$ as the ECL indicator was intercalated into the grooves of supersandwich DNA structure. The ECL intensity was corresponding to the concentration of the HIV-1 gene with a detection limit of 0.022 pM.

12.3.3 Fluorescence Supersandwich Assays

Luminescent silver nanoclusters (AgNC) synthesized using DNA as scaffolds could be acted as fluorescent labels [34]. Wang et al. fabricated a supersandwich DNA/AgNC luminescent sensor through the artificial oligonucleotide scaffold with AgNC biomineralizing unit and target DNA recognizing unit [35]. The recognizing unit

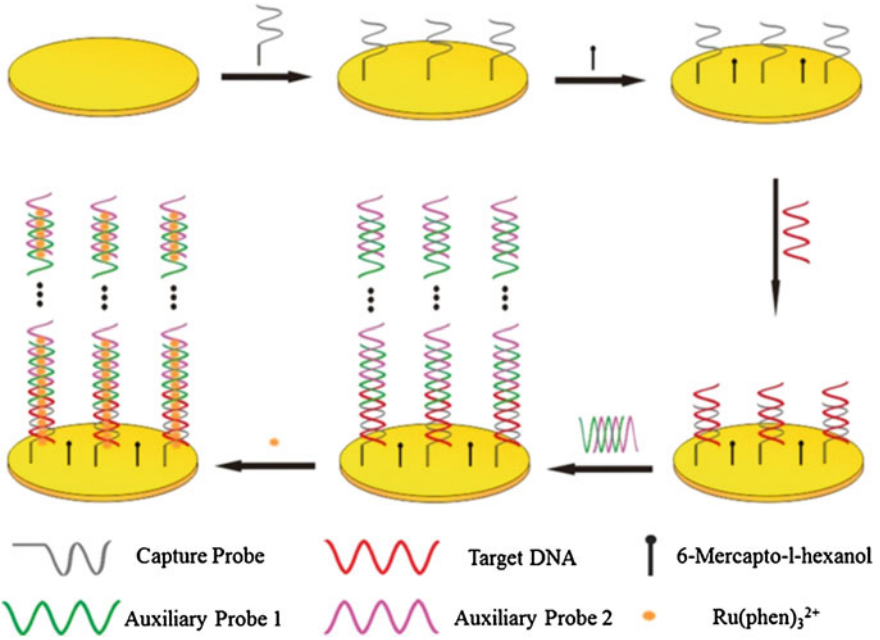

Fig. 12.5 Schematic illustration of an ECL supersandwich assay for the detection of the human immunodeficiency virus-1 (HIV-1) gene. The high sensitivity and selectivity of electrochemiluminescence DNA biosensor can be largely improved by using supersandwich dsDNA along with ECL indicators. (Reprinted with the permission from Ref. [33]. Copyright 2014 Springer-Verlag Wien)

hybridized with the target DNA to create supersandwich DNA structure. The luminescence intensity of AgNC was relative to the concentration of the target DNA. A supersandwich fluorescence in situ hybridization strategy for the detection of mRNA at the single-cell level was reported recently, as shown in Fig. 12.6 [36]. Three kinds of mRNA were tested. Taking TK1 mRNA as an example, a DNA probe entered the fixed cells and hybridized with the target mRNA. Two fluorescent signal probes were hybridized to form long concatamers, thus amplifying the signal of the target mRNA.

12.3.4 Surface Plasmon Resonance Supersandwich Assays

Surface plasmon resonance (SPR) is a powerful technology for label-free, real-time, and in situ detection of biomarkers [37]. Surface plasmon resonance biosensor for label-free detection of miRNA based on supersandwich DNA structure and streptavidin signal amplification has been developed by Ding et al. in 2014 [38]. The capture DNA probes immobilized on the gold electrode selectively captured the target miRNA to form supersandwich DNA structure and then hybridized streptavidin through biotin binding for signal amplification, thus leading to the increase of the SPR signal. The assay showed high sensitivity with a detection limit of

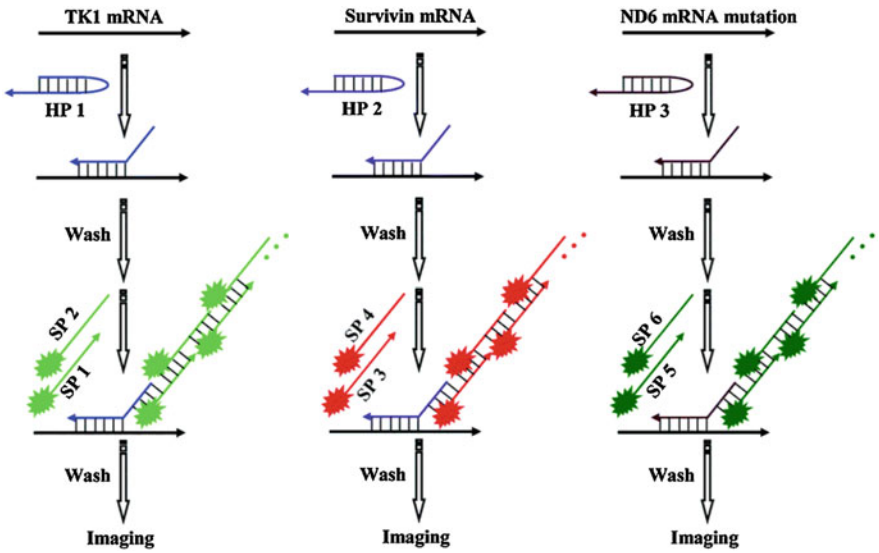

Fig. 12.6 Schematic illustration of a fluorescence supersandwich assay for the detection of mRNA. Two fluorophore-labeled signal probes are used to generate a supersandwich product, which in turn generates numerous signal probes located at the target mRNA position, resulting in the in situ fluorescence signal amplification. (Reprinted with the permission from Ref. [36]. Copyright 2016 The Royal Society of Chemistry)

miRNA down to 9 pM. Surface plasmon resonance biosensor for enzyme-free detection of miRNA based on supersandwich DNA structure and gold nanoparticles has been proposed by Wang et al. in 2016, as shown in Fig. 12.7 [39]. The capture DNA with a loop immobilized on the gold film surface captured miRNA-21. DNA-linked AuNPs were then captured by hybridization and the report DNAs were hybridized starting from DNA-linked AuNPs to form supersandwich DNA structure, which enhanced the shift of resonance angle. This assay showed high selectivity for the discrimination of single-base mismatch and detected ca. 8 fM miRNA-21. They then lowered a detection limit of miRNA-21 to ca. 0.6 fM by further increase of SPR response using AgNPs absorbed into the grooves of supersandwich DNA structure as additional signal amplification tool [40].

12.4 Supersandwich Assays for Small-Molecule and Ion Detection

12.4.1 Electrochemical Supersandwich Assays

Adenosine triphosphate (ATP), a small molecule generally acknowledged as a major cellular energy currency, plays an important role in most enzymatic activities

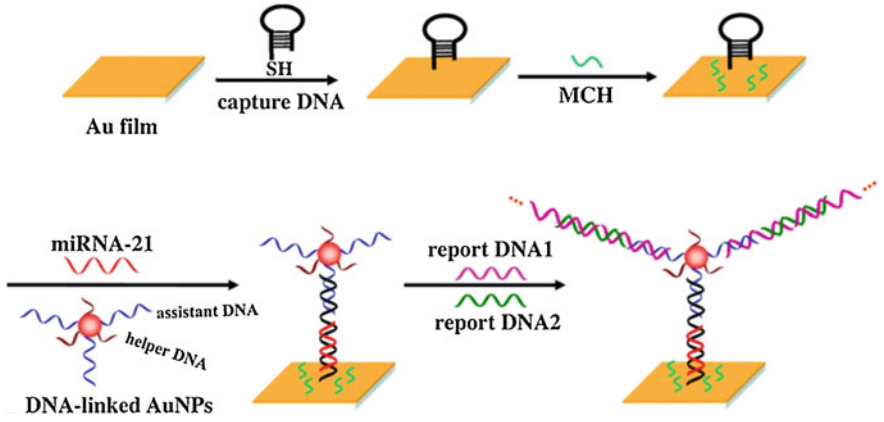

Fig. 12.7 Schematic illustration of a surface plasmon resonance supersandwich assay for the detection of miRNA. The loop capture DNA immobilized on the Au film surface captures miRNA-21, and DNA-linked AuNPs are then hybridized. The report DNA1 and report DNA2 are introduced to form supersandwich DNA structure. (Reprinted with the permission from Ref. [39]. Copyright 2015 Elsevier)

[41]. ATP depletion is a key process in pathogenesis, particularly, Parkinson's disease, hypoglycemia, and hypoxia [42]. Therefore, the detection of ATP is not only of research interest but of clinical importance [43]. Xia et al. developed an electrochemical aptasensor based on a dual-signaling strategy and a supersandwich assay for the detection of ATP, as shown in Fig. 12.8 [44]. The capture probe anchored on the surface of the gold electrode hybridized with methylene blue (MB)-labeled signal probe and ferrocene (Fc)-modified signal probe to create supersandwich DNA structure. In the presence of ATP, supersandwich DNA structure would disassemble because ATP bounds its aptamer, resulting in the release of the signal probes to generate the reduction signals of MB and Fc. Taking dual signals as the response signal, ATP was detected at a detection limit of 2.1 nM. They also constructed supersandwich DNA structure in the nanopores for the detection of ATP [32, 45]. Of note, other small molecules such as adenosine [46] and cisplatin [47] were also detected by supersandwich electrochemical biosensors with excellent sensitivity and reproducibility.

Mercury(II) ion (Hg^{2+}) specifically combines with two thymine bases (T) to afford stable T-Hg^{2+}-T bases pairs [48]. Wang et al. fabricated supersandwich DNA assay based on T-Hg^{2+}-T to amplify the electrochemical signal for the detection of Hg^{2+} with a detection limit of 10 fM [49]. Silver ion (Ag^{+}), a highly toxic heavy metal ion, has caused serious health and environment attention in recent years [50]. Similar to T-Hg^{2+}-T, Ag^{+} specifically combines with two cytosine base (C) to form stable C-Ag^{+}-C bases pairs [51]. Supersandwich electrochemical biosensor based on magnetic nanoparticles labeling with hybridization chain reaction amplification triggered by C-Ag^{+}-C was developed for the detection of Ag^{+} with a detection limit of 0.5 fM [52]. Recently, the combination of supersandwich DNA structure and

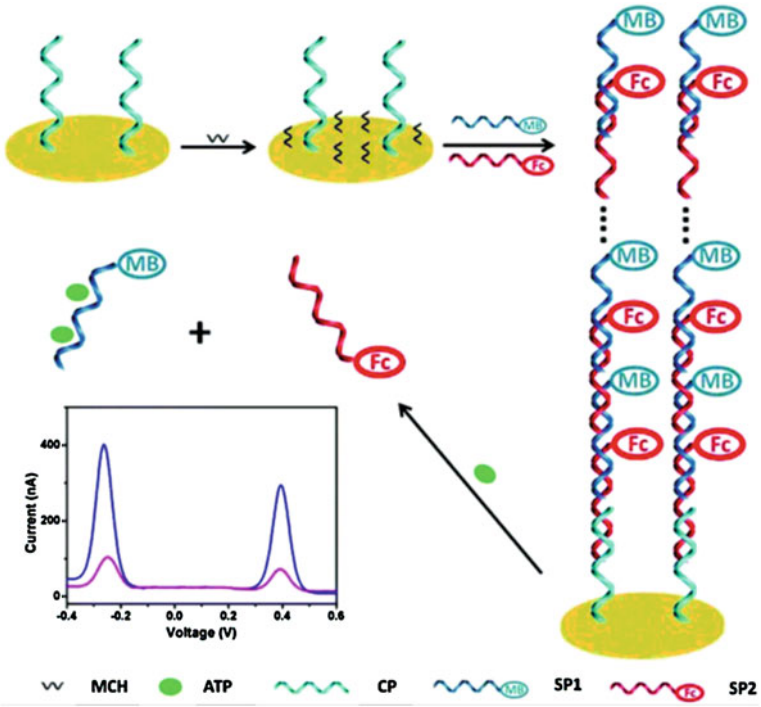

Fig. 12.8 Schematic illustration of an electrochemical supersandwich assay for the detection of ATP. The capture probe is anchored at the gold electrode surface and then combines with methylene blue (MB)-modified signal probe 1 (SP1) and ferrocene (Fc)-labeled signal probe 2 (SP2) to form supersandwich DNA structure. (Reprinted with the permission from Ref. [44]. Copyright 2016 The Royal Society of Chemistry)

Zn^{2+}-requiring DNAzymes in the nanopores provided a strategy to detect Zn^{2+} with a detection limit of 1 nM [53].

12.4.2 Electrochemiluminescence Supersandwich Assays

Yuan et al. demonstrated a supersandwich ECL assay for the detection of ochratoxin A (OTA), as shown in Fig. 12.9 [54]. The capture probe immobilized on the surface of the gold electrode triggered a cross-opening process of two hairpin DNAs to form supersandwich DNA structure. Hemin induced the formation of hemin/G-quadruplex DNAzyme structure. In the presence of the target OTA and $RecJ_f$ exonuclease, supersandwich DNA structure disassembled to generate a significant ECL signal of the $O_2/S_2O_8^{2-}$ system. This assay achieved a detection limit of 75 fg mL^{-1} for the detection of OTA. Xu et al. developed a label-free supersandwich ECL assay based on T-Hg^{2+}-T coordination and the intercalation of Ru

$(phen)_3^{2+}$ for the detection of Hg^{2+} [55]. This assay achieved a detection limit of 0.25 nM for the detection of Hg^{2+}, meeting the requirement of U.S. Environmental Protection Agency for Hg^{2+} in drinkable water (<10 nM).

12.4.3 Fluorescence Supersandwich Assays

ATP was detected using a label-free fluorescence strategy based on the ligation-triggered supersandwich that was reported by Yang et al., as shown in Fig. 12.10 [56]. First, a dsDNA probe was designed as the substrate of ATP-dependent ligation. SYBR Green I (SG I) as the readout signal was intercalated into the grooves of the dsDNA probe. With the addition of ATP, the recognition of T4 DNA ligase caused the dsDNA probe formed supersandwich DNA structure, resulting in the enhancement of the fluorescence signal. This assay showed a high sensitivity with a detection limit of 200 pM for the detection of ATP. For the detection of Hg^{2+}, Xu et al. demonstrated a label-free supersandwich fluorescence assay based on the generation of supersandwich DNA structure by T-Hg^{2+}-T with a detection limit of 2.5 nM [57]. Genefinder (GF) intercalated into the grooves of dsDNA was employed as the readout fluorescence signal.

12.5 Supersandwich Assays for Cell Detection

The detection of cancer cells has become an increasingly important topic for monitoring the progressions of diseases and diagnosing cancers [58]. Zhu et al. developed a supersandwich assay through signal amplification for the detection of

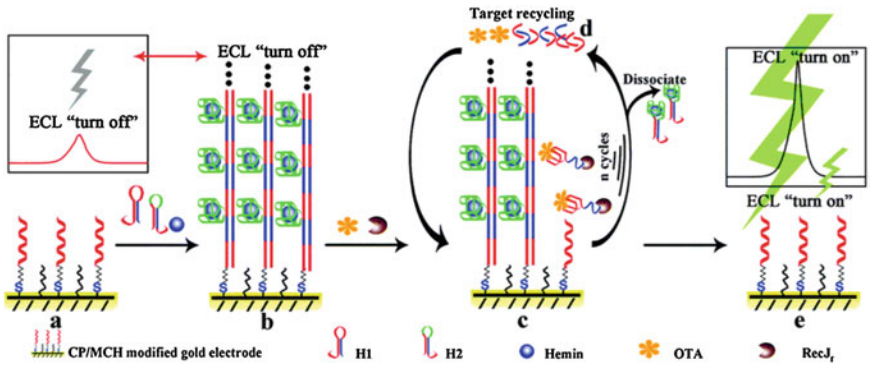

Fig. 12.9 Schematic illustration of an ECL supersandwich assay for the detection of small molecule OTA. The supersandwich DNA structure is formed on capture probes/6-mercapto-1-hexanol (CP/MCH)-modified gold electrode through a cross-opening process of the two hairpin DNAs. The hemin/G-quadruplex DNAzyme nanostructures are formed upon addition of hemin, the target OTA and $RecJ_f$ exonuclease. (Reprinted with the permission from Ref. [54]. Copyright 2014 The Royal Society of Chemistry)

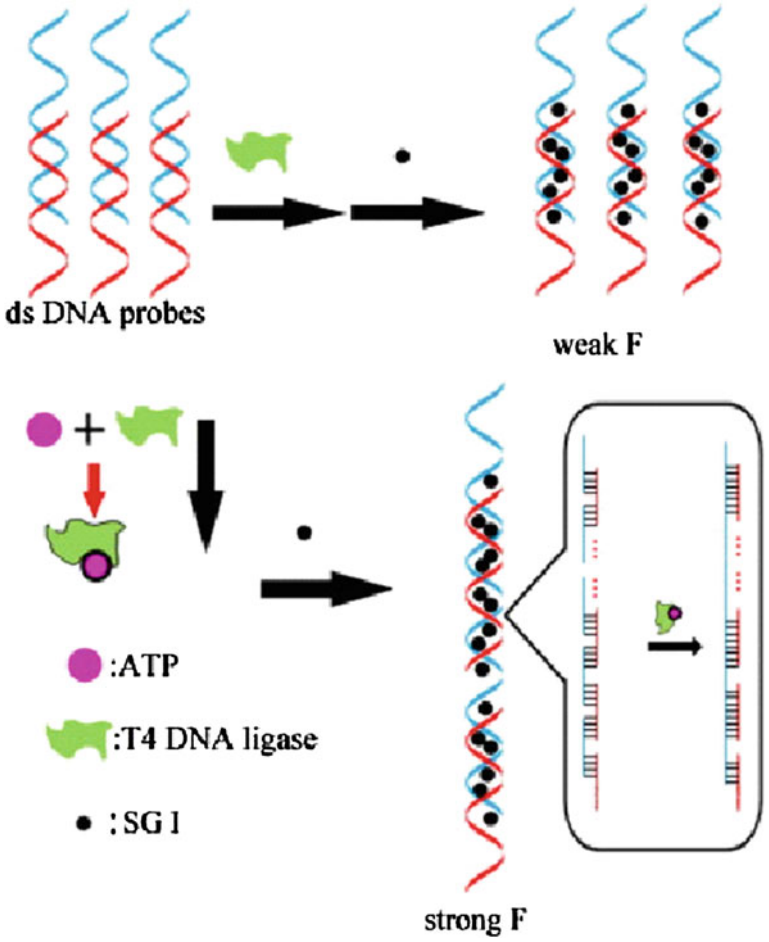

Fig. 12.10 Schematic illustration of a fluorescence supersandwich assay for the detection of ATP. The double-stranded DNA (dsDNA) probes form a supersandwich which can be detected using dsDNA-specific fluorescent SYBR Green I (SG I). (Reprinted with the permission from Ref. [56]. Copyright 2014 Elsevier)

cancer cells, as shown in Fig. 12.11 [59]. Aptamer-DNA concatamer-quantum dots probes were fabricated by the hybridization of aptamer-DNA and quantum dot-modified DNA with the capture DNA. Multiwall carbon nanotubes (MWCNTs), polydopamine (PDA), and gold nanoparticles (AuNPs) were employed to fabricate the electrode material MWCNTs@PDA@AuNPs through a layer-by-layer method. Concanavalin A (Con A) was captured by multiwall carbon nanotubes@polydopamine@gold nanoparticles (MWCNTs@PDA@AuNPs) that were absorbed on the surface of glassy carbon electrode (GCE). After cancer cells (CCRF-CEM cells) were captured by Con A, aptamer-DNA concatamer-quantum

dots probes were modified through the specific recognition of the aptamer to cancer cells. CCRF-CEM cells were detected by both fluorescence and electrochemical methods. The signal amplification of the DNA concatamer and quantum dots improved the sensitivity with a detection limit of 50 cells mL^{-1}. In addition, this assay could differentiate cancer cells from normal cells. A supersandwich electrochemical assay based on G-quadruplex DNAzyme and a supersandwich surface plasmon resonance assay using multiple signal amplification strategy were reported for the detection of cancer cells [40, 60]. Furthermore, circulating tumor cells (CTCs), a kind of tumor cells in the peripheral blood, were detected through a

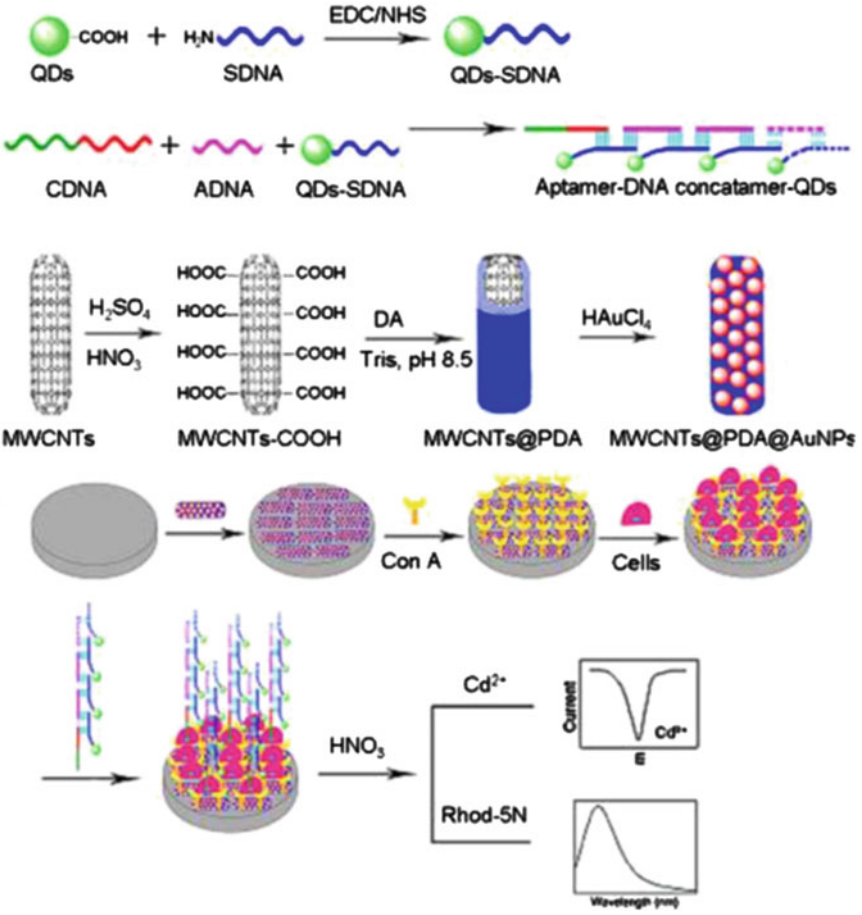

Fig. 12.11 Schematic illustration of a supersandwich assay for the detection of cancer cells. The DNA concatamer-QDs are designed via DNA hybridization. MWCNTs@PDA@AuNPs composites are assembled to the electrode for immobilization of concanavalin A (Con A). CCRF-CEM cancer cells are selected with aptamer-DNA concatamer-QDs as probes. (Reprinted with the permission from Ref. [59]. Copyright 2013 American Chemical Society)

supersandwich electrochemical assay using signal amplification strategy with a detection limit of 10 cells mL^{-1} [61].

12.6 Conclusion

In this chapter, we have summarized the recent development of several biosensors based on supersandwich assays for the detection of proteins, nucleic acids, small molecules, ions, and cells. It is clear that each strategy has its features and limitations. For instance, supersandwich electrochemical assays can detect the target with good sensitivity, but electrochemical detecting instruments are usually required. A facile and sensitive biosensor based on supersandwich assays without complex operations and professional instrumentation will be popularly achieved as immunochromatographic strip. We are happy to see that biosensors based on supersandwich assays step out of the laboratory to the house in the future.

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