Structure and Immobilization of Aptamers

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Contributor: Lianxi Zheng , Hibba Yousef , Yang Liu

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label free thrombin aptasensor electrochemical

1. Introduction

Thrombin is a serine protease involved in hemostasis [1], converting the precursor fibrinogen into fibrin for the formation of blood clots [2]. Thrombin is not present in the blood under normal conditions, but rather its precursor, prothrombin, can be found in circulation [3]. Upon exposure of tissue factor to blood, the coagulation cascade is activated to generate thrombin from prothrombin at the site of injury, occurring in a precise and balanced manner [4]. Thrombin plays a central role in hemostasis as it converts fibrinogen into fibrin, which then is deposited to entrap aggregated platelets and stabilize the blood clot [5]. Imbalances in coagulation, including the overexpression of thrombin, can lead to various pathologies [2] such as heart attack, stroke, liver disease, deep vein thrombosis, leukemia, and pulmonary embolism, among many more [6][7]. It is reported that COVID-19 patients have elevated thrombin levels on diagnosis [8], involved in a condition known as COVID-19-associated coagulopathy (CoAC), which manifests as numerous thromboembolic complications in COVID-19 patients [9]. Consequentially, it is of clinical significance to develop a fast and accurate method for the quantitative detection of thrombin [2].

In general, the detection of any specific bio-molecule needs a biological recognition element that has a strong affinity to the target [10]. Antibodies have typically been employed as a biorecognition element due to their high affinity to a large range of molecules. However, antibody-based biosensors have suffered from low sensitivity and have therefore not been widely adopted given the fact that biomarkers of most diseases are present only in trace amounts in the bloodstream. Although immunosensors with improved sensitivity have been developed, their tedious and complicated fabrication processes, as well as their enhanced cost, have impeded their practical applications [11].

Electrochemical aptasensors, which benefit from the extraordinary selectivity of aptamers and the high sensitivity of the cost-effective electrochemical techniques, have attracted considerable interest since the pioneering work on protein detection was reported by Kazunori et al. [12]. Nucleic acids (NAs), typically known for their storage and translation of genetic materials, have the potential to perform a more diverse range of functions, including the use as aptamers with a remarkable ability to bind their targets, for the construction of electrochemical aptasensors [13]. Aptamers offer numerous advantages over antibodies as a biorecognition element for biosensing, particularly in

terms of their simplicity and low production cost without the requirement of cell or animal lines [14]. Additionally, aptamers can bind to a wide variety of molecules [15], including targets for which antibodies are difficult to obtain [16]. Aptamers can also be easily modified with functional groups [17], whereas this functionalization process in antibodies is not site-specific, which could result in the alteration of target-binding areas, thus interfering with their activity [18]. Furthermore, aptamers are superior in thermal and conformational stability, being able to recover their original configurations even after denaturation [18]. Among various analytical methods, electrochemical techniques are attractive when applied to aptasensing due to the high sensitivity, fast response, small sample consumption, and low cost. In addition, the capability of electrochemical aptasensors in miniaturization and mass production makes them promising for point-of-care applications [19][20][21].

2. Structure and Immobilization of Aptamers

2.1. Structure and Function of Thrombin Aptamers

Aptamers are synthetically produced via a process known as systematic evolution of ligands by exponential enrichment (SELEX) [22]. The process was initially reported in 1990 [23][24] and has since been used to generate thousands of aptamers against a wide variety of target molecules [25][26]. SELEX is an iterative process, involving multiple repetitive steps [27]. Libraries containing over 10^{15} random oligonucleotide sequences are screened for specific sequences with a high affinity to a determined analyte. These sequences are then amplified via reverse transcription–polymerase chain reaction (PCR), allowing for the domination of the sequences with the highest affinity [28]. The affinity between an aptamer and its analyte is characterized by a value known as the dissociation constant (K_d), with lower K_d values reflective of stronger binding [13]. Iterative cycles of selection and amplification are carried out in order to narrow down the best candidate for the specified analyte. Subsequently, an analyte-specific aptamer is identified [28].

The first thrombin aptamer was isolated in 1992 by Bock et al. [29], which was the first single-stranded DNA oligonucleotide considered for use as an aptamer. This 15 nucleotide sequence (5′- GGTTGGTGTGGTTGG-3′) has been extensively studied as an anticoagulant therapeutic agent and is commonly known as the thrombin-binding aptamer (TBA) [7]. Two aptamers for α thrombin have been synthesized, Apt29 and Apt15, consisting of 29 and 15 nucleotides, respectively [30]. These non-B DNA sequences are able to form secondary, non-canonical structures and do not follow Watson-Crick base pairing [31]. Rich in the nucleotide Guanine, structures, known as G-quartets, are assembled in which four guanine nucleotides form a planar structure via Hoogsteen hydrogen bonds, stabilized at their center by a monovalent or bivalent cation which acts to partially reduce the repulsion between the negatively charged nucleic acids [32][33][34]. G-quartets can further assemble into antiparallel G-quadruplexes (G4) by forming π-π bonds between quartets in a stack-like formation [35]. Antiparallel G4 is a highly organized, chair-like structure, which is the signature structure of TBAs [36].

thrombin contains two positively charged binding sites, known as exosite I (Fibrinogen-binding) and exosite II (Heparin-binding), to which negatively charged complementary regions of the aptamers will bind. Apt29 binds to thrombin at its heparin-binding exosite, whereas Apt15 binds to the fibrinogen-binding exosite, mediated by van der

Waals forces and hydrogen bonds [3][37]. Upon binding to thrombin, the aptamer will undergo conformational changes via three-dimensional intramolecular folding, converting from a hairpin to tertiary G4 structure, which is stabilized by thrombin [38].

The presence of two different binding sites in thrombin provides a unique advantage in its detection. This allows the employment of a technique known as a sandwich assay, in which two aptamers can simultaneously bind to thrombin, therefore enhancing the detection sensitivity [39]. Traditionally, aptamer-antibody assays have been used in the sandwich format. However, problems such as antibody instability and the high cost of this technique have been resolved by the use of aptamer-pair based detection in its place [39]. Very few kinds of proteins other than thrombin have the advantage of two aptamer binding sites [40].

2.2. Immobilization of Aptamers

Immobilization of aptamers onto the surface of the electrode is an essential step in the construction of an electrochemical aptasensor [41], where maximizing surface density and maintaining the binding function of the aptamers are primary concerns [42]. The simplest method of immobilization is the self-assembly of thiol-modified aptamers onto gold electrodes through covalent bonding [43], which leads to the formation of SAMs on the electrode surface [44]. Other methods of immobilization include surface modification of the electrode along with the addition of a functional group to the 3′ or 5′ end of the aptamers [41]. The availability of chemical groups on the surface of the modified electrode, such as hydroxyl or carboxyl groups, allows for covalent interaction with an amino-modified aptamer [43], as an example. In general, physical adsorption is not recommended, as desorption of aptamers from the surface leads to instability [45]. Further simplified immobilization can be achieved by modifying the electrode surface with carbon nanotubes (CNT) [42] or graphene [46], eliminating the need for functional group modification of aptamers, as these materials can bind directly to aptamers through π-π stacking.

Nonspecific adsorption of proteins to the electrode surface could cause a biofouling problem and also have a dramatic effect on the folding and orientation of immobilized aptamers, and should therefore be minimized [47]. Bovine serum albumin (BSA) [48] or mercapto-hexanol (MCH) can be used to mitigate such problems by blocking the surface that has not been occupied by aptamers [43]. In addition, hydrophilic materials have been used to increase the hydrophilicity of the electrode surface, further increasing resistance to biofouling [49]. More specific details will be discussed in the following sections according to the electrode materials used in aptasensors.

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