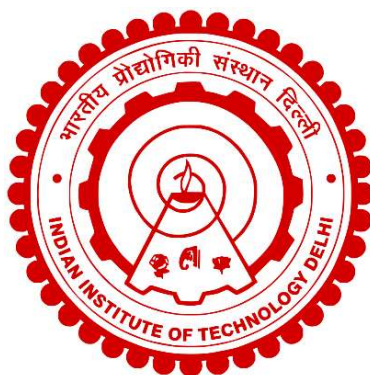


DESIGN, DEVELOPMENT, AND METHOD EVALUATION OF APTAMER-BASED AFLATOXIN DETECTION

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**CENTRE FOR RURAL DEVELOPMENT AND TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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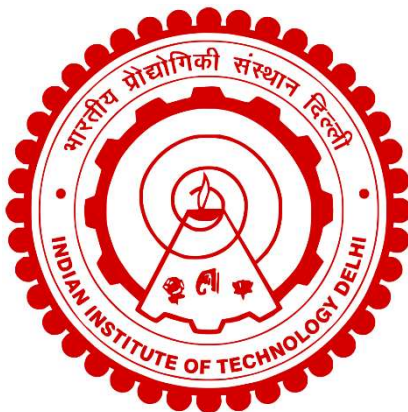
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Submitted

In fulfilment of the requirements for the degree of

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*Dedicated to Mumma and
Papa, my foundation...*

Certificate

This is to certify that the thesis entitled " **Design, Development, and Method Evaluation of Aptamer-based Aflatoxin Detection**" being submitted by **Ms. Shazia Shareef** to the Indian Institute of Technology Delhi for the award of "**Doctor of Philosophy**" is a record of Bonafide research work carried out by her. She has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis. To the best of my knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree or diploma.

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Abstract

Aflatoxins are the carcinogenic secondary metabolites produced by fungi such as *Aspergillus flavus* and *A. parasiticus*. These fungi are widely spread in nature and have severely contaminated food supplies for humans and animals. Consuming food or feed contaminated with aflatoxins can lead to hepatocellular carcinoma, immunosuppression, growth retardation, acute hepatic toxicity, reproductive toxicity, malnutrition, and increased susceptibility to infections and liver cirrhosis. Therefore, the detection and quantification of aflatoxins in foods and feeds are critical aspects of safety and health concerns. The challenges are (a) Aflatoxins are low molecular weight, non-immunogenic, monoepitopic antigens, and (b) Their concentration in contaminated food/feed is very low (ppb to ppm). The primary strategy to eliminate Aflatoxin from food/feed is to eliminate the contaminated commodities through detection at various levels of the food supply chain. Several methods are currently being used to detect Aflatoxin in food/feeds, which include Thin Layer Chromatography (TLC), High-performance liquid chromatography (HPLC), Liquid chromatography – Mass spectroscopy (LC-MS) and Antibody-based techniques [Enzyme-linked immunosorbent assay (ELISA) and Radioimmunoassay (RIA)]. Though TLC is a comparatively simpler and cheaper method, it is not specific to anyone analyte. It required sample cleaning, processing, and derivatization, as well as a precise mobile phase and a UV cabinet to visualize the results and is referred for only qualitative analysis. Hence, HPLC and LC-MS are more accurate in quantitative analysis. However, their initial investment, maintenance and running costs are high. Requires sample preparation and purification stages. Skilled labour is required to handle the instrument and interpret the results. HPLC and LC-MS are highly recommended to laboratories. ELISA is both quantitative and qualitative in nature and can be used at the field level. However, the cost of the ELISA kits is still considered higher as they involve antibody production and a cold supply chain.

In the recent past, DNA-based aptamers have been gaining attention in the area of biosensors. **Aptamers** are short oligonucleotides of 20 – 80 bases. They function similarly to antibodies by forming a three-dimensional structure, enabling specific and highly sensitive interactions with the target analyte. Once the aptamer generated against a target analyte through the **systemic evolution of ligands by exponential enrichment (SELEX)** process, they can be easily reproduced. Moreover, as DNA based aptamers are stable at high temperatures, aptamers-based kits can be stored and transported at room temperature. In addition, these aptamers are highly versatile and robust, which

can be used to develop various types of assays and kits without compromising sensitivity and specificity.

In this regard, the present study focused on developing aflatoxin-specific aptamers with high affinity and specificity. In the first phase of the study, **AuNPs-based SELEX** procedure performed to screen and evaluate aptamers specific to Aflatoxin B₁ (AFB₁). The SELEX cycles involved systematic optimization of ssDNA library enrichment using AuNP-based colorimetric assays for real-time monitoring.

Following this, **next-generation sequencing (NGS)** was performed to analyze aptamer pools enriched through AuNP-based SELEX. Two methodologies were employed: **MiSeq NGS** for the 12th SELEX cycle pool following vector-based library preparation and **NovaSeq NGS** for direct sequencing of 4th, 8th, 12th, and 15th cycle pools. The raw reads quality control analysis, merging reads, and adapter removal, were conducted using FastQC, Trimmomatic, and AptaSUITE tools. MiSeq sequencing identified clusters of aptamer sequences, while NovaSeq revealed 22 motifs. Motif analysis using MEME and MAST further refined aptamer selection.

Further, the sensitivity, specificity, and binding characteristics of top **17 DNA aptamers** were evaluated against AFB₁. Using **AuNP-based colorimetric assay**, the dissociation constants (K_d) of the aptamer-AFB₁ complexes were determined, revealing robust binding affinities even at nanomolar concentrations for AFB₁. The top four aptamers with the lowest K_d values **CPMA1, L1, L2, and L3** were shortlisted for further analysis. **Circular Dichroism (CD) Spectropolarimetry** demonstrated conformational changes in aptamers upon AFB₁ binding, indicating stable aptamer-AFB₁ complex formation. **Fluorescence-based assays**, including a SyBr Green-I label-free approach and a graphene oxide (GO) and FAM-labelled aptamer assay, further confirmed the strong binding kinetics of these aptamers. **Specificity** and **cross-reactivity** studies using AuNPs-based assay revealed minimal binding to non-target analytes, including AFB₂, AFG₁, AFG₂, AFM₁, Ochratoxin A, Fumonisin B₁, and Bovine Serum Albumin (BSA), demonstrating high specificity towards AFB₁.

Additionally, computational methods performed to analyze the binding specificity and stability of ssDNA selected aptamers towards AFB₁. **Secondary structures** of aptamers were predicted using the MFold webserver, revealing diverse folding patterns. The tertiary structures were modeled with RNA Composer and refined to DNA-like configurations. **Molecular docking** with AutoDock tools demonstrated strong binding affinities, with aptamer L1 showing the lowest binding

energy (-5.06 kcal/mol). **Molecular dynamics simulations** in GROMACS validated the stability and dynamics of aptamer-AFB₁ complexes over 100 ns, with RMSD, RMSF, Rg, SASA, and hydrogen bond analyses confirming equilibrium and interaction stability.

In the final objective, a rapid, on-site, and cost-effective **lateral flow assay (LFA)** was developed for the detection of AFB₁ using the **L2 aptamer** and complementary cDNA-thiol-gold nanoparticles (cDNA-SH-AuNPs) as colorimetric probes, with poly (diallyl dimethylammonium chloride) (PDDA) incorporated at the control line. The assay followed a dual-line detection format: in the absence of AFB₁, cDNA-SH-AuNPs hybridize with the biotinylated aptamer at the test line and bind at the PDDA control line, resulting in visible signals at both lines. In the presence of AFB₁, its specific binding to the aptamer disrupts the aptamer-cDNA interaction, preventing capture at the test line and allowing the cDNA-SH-AuNPs to migrate and bind only at the control line, producing a single visible signal. This design provides an internal validation mechanism, ensuring proper sample migration and reagent functionality. The assay exhibited high specificity with negligible cross-reactivity to other mycotoxins. **A limit of detection (LOD) of 0.648 μ M** and a **sensitivity range of 0.01–0.2 μ M (~3–6 ppb)** were achieved, indicating its suitability for detecting AFB₁ even at low concentrations. Successful detection of AFB₁ in spiked maize and groundnut samples confirmed the assay's practical applicability, establishing it as a sensitive, reliable, and field-deployable tool for monitoring AFB₁ contamination in food and feed commodities.

Overall, this study successfully developed highly specific and sensitive DNA-based aptamers for the detection of AFB₁ using a combination of AuNP-based SELEX, next-generation sequencing, and computational analysis. The resulting aptamers were applied to create a rapid and cost-effective LFA, which demonstrated robust performance in detecting AFB₁ in real spiked samples, offering a practical solution for monitoring aflatoxin contamination in food and feed commodities.

सार

अप्लाटॉक्सिन कार्सिनोजेनिक माध्यमिक मेटाबोलाइट्स हैं, जो *एस्पेरगिलस फ्लेवस* और *ए. पैरासिटिकस* जैसे कवकों द्वारा उत्पन्न होते हैं। ये कवक प्रकृति में व्यापक रूप से फैले हुए हैं और उन्होंने मनुष्यों और पशुओं के खाद्य आपूर्ति को गंभीर रूप से दूषित किया है। अप्लाटॉक्सिन से दूषित खाद्य या चारे के सेवन से हेपाटोसेलुलर कार्सिनोमा (लिवर कैंसर), इम्यूनोसप्रेसन, विकास अवरोध, तीव्र यकृत विषाक्तता, प्रजनन विषाक्तता, कुपोषण, संक्रमण के प्रति बढ़ी हुई संवेदनशीलता और लिवर सिरोसिस जैसी समस्याएं हो सकती हैं। इसलिए, खाद्य और चारे में अप्लाटॉक्सिन की पहचान और मात्रात्मक विश्लेषण करना सुरक्षा और स्वास्थ्य संबंधी चिंताओं का एक महत्वपूर्ण पहलू है। अप्लाटॉक्सिन के संदर्भ में कुछ चुनौतियां हैं: (a) अप्लाटॉक्सिन कम आणविक भार वाले, गैर-प्रतिरक्षात्मक और मोनोएपिटोपिक एंटीजन हैं। (b) दूषित खाद्य/चारे में इनकी सांद्रता बहुत कम होती है (पीपीबी से पीपीएम स्तर तक)। अप्लाटॉक्सिन को खाद्य/चारे से समाप्त करने की प्राथमिक रणनीति यह है कि दूषित वस्तुओं की पहचान कर उन्हें खाद्य आपूर्ति श्रृंखला के विभिन्न स्तरों पर अलग किया जाए। वर्तमान में अप्लाटॉक्सिन का पता लगाने के लिए कई विधियों का उपयोग किया जा रहा है, जिनमें शामिल हैं: थिन लेयर क्रोमैटोग्राफी, हाई-परफॉर्मेंस लिक्विड क्रोमैटोग्राफी, लिक्विड क्रोमैटोग्राफी-मास स्पेक्ट्रोस्कोपी, और एंटीबॉडी-आधारित तकनीकें जैसे [एंजाइम-लिंकड इम्यूनोसॉरबेंट असे और रेडियोइम्यूनोअसे]। हालांकि, थिन लेयर क्रोमैटोग्राफी तुलनात्मक रूप से सरल और सस्ती विधि है, लेकिन यह किसी एक विशिष्ट एनालाइट के लिए सटीक नहीं है। इसमें नमूनों की सफाई, प्रोसेसिंग और डेरिवेटाइजेशन की आवश्यकता होती है, साथ ही एक सटीक मोबाइल फेज और परिणामों को देखने के लिए एक यूवी कैबिनेट चाहिए। इसे केवल गुणात्मक विश्लेषण के लिए उपयोग किया जाता है। इसलिए, हाई-परफॉर्मेंस लिक्विड क्रोमैटोग्राफी और लिक्विड क्रोमैटोग्राफी-मास स्पेक्ट्रोस्कोपी मात्रात्मक विश्लेषण में अधिक सटीक हैं। हालांकि, इनकी शुरुआती लागत, रखरखाव और संचालन खर्च अधिक हैं। नमूनों की तैयारी और शुद्धिकरण के चरणों की आवश्यकता होती है। उपकरण को संभालने और परिणामों की व्याख्या के लिए कुशल श्रमिकों की आवश्यकता होती है। ये विधियां प्रयोगशालाओं के लिए अत्यधिक अनुशंसित हैं। एंजाइम-लिंकड इम्यूनोसॉरबेंट असे मात्रात्मक और गुणात्मक दोनों प्रकार का विश्लेषण प्रदान करती है और इसे क्षेत्र स्तर पर उपयोग किया जा सकता है। हालांकि, ELISA किट की लागत अभी भी अधिक मानी जाती है क्योंकि इसमें एंटीबॉडी उत्पादन और कोल्ड सप्लाय चैन शामिल है।

हाल के वर्षों में, डीएनए-आधारित एष्टामर बायोसेंसर्स के क्षेत्र में विशेष ध्यान आकर्षित कर रहे हैं। एष्टामर 20-80 बेस लंबे छोटे ओलिगोन्यूक्लियोटाइड्स होते हैं। ये एंटीबॉडी के समान कार्य करते हैं, क्योंकि ये

एक त्रि-आयामी संरचना बनाते हैं, जो लक्ष्य एनालाइट के साथ विशिष्ट और अत्यधिक संवेदनशील इंटरैक्शन को सक्षम बनाती है। जब एक लक्ष्य एनालाइट के खिलाफ एण्टामर का उत्पादन सिस्टमेटिक इवोल्यूशन ऑफ लिगेण्ड्स बाय एक्सपोज़िशन एनरिचमेंट (SELEX) प्रक्रिया के माध्यम से किया जाता है, तो इन्हें आसानी से पुनः उत्पादित किया जा सकता है। इसके अलावा, डीएनए-आधारित एण्टामर उच्च तापमान पर स्थिर रहते हैं, जिससे एण्टामर-आधारित किट्स को कमरे के तापमान पर संग्रहीत और परिवहन किया जा सकता है। साथ ही, ये एण्टामर अत्यधिक बहुमुखी और मजबूत होते हैं, जिन्हें विभिन्न प्रकार के परीक्षणों और किटों को विकसित करने के लिए उपयोग किया जा सकता है, वह भी संवेदनशीलता और विशिष्टता से समझौता किए बिना।

इस संदर्भ में, वर्तमान अध्ययन का उद्देश्य उच्च अभिप्रेरणा और विशिष्टता के साथ अफ्लाटॉक्सिन-विशिष्ट एण्टामर विकसित करना था। अध्ययन के पहले चरण में, अफ्लाटॉक्सिन B₁ (AFB₁) के लिए एण्टामर की स्क्रीनिंग और मूल्यांकन करने के लिए AuNPs-आधारित SELEX प्रक्रिया को लागू किया गया। SELEX चक्रों में AuNP-आधारित कलोरिमेट्रिक assays का उपयोग करके ssDNA पुस्तकालय को समर्पित रूप से समृद्ध करना और वास्तविक समय में निगरानी के लिए इसके अनुकूलन की प्रक्रिया शामिल थी।

इसके बाद, AuNP-आधारित SELEX के माध्यम से समृद्ध किए गए एण्टामर पूल्स का विश्लेषण करने के लिए नेक्स्ट-जनरेशन सीक्वेंसिंग (NGS) किया गया। दो पद्धतियों का उपयोग किया गया: MiSeq NGS का उपयोग 12वें SELEX चक्र पूल के लिए किया गया, जिसमें वेक्टर-आधारित पुस्तकालय तैयारी का पालन किया गया और NovaSeq NGS का उपयोग 4वें, 8वें, 12वें और 15वें चक्र पूल्स की सीधे सीक्वेंसिंग के लिए किया गया। कच्ची पढ़ाई की गुणवत्ता नियंत्रण विश्लेषण, पढ़ाई का विलय, और अडैप्टर हटाने का कार्य FastQC, Trimmomatic, और AptaSUITE उपकरणों का उपयोग करके किया गया। MiSeq सीक्वेंसिंग ने एण्टामर अनुक्रमों के क्लस्टर पहचाने, जबकि NovaSeq ने 22 मोटिफ़्स का खुलासा किया। MEME और MAST का उपयोग करके मोटिफ़ विश्लेषण ने एण्टामर चयन को और परिष्कृत किया।

इसके अलावा, शीर्ष 17 डीएनए एप्टामर्स की संवेदनशीलता, विशिष्टता और बाइंडिंग विशेषताओं का मूल्यांकन AFB₁ के खिलाफ किया गया। AuNP-आधारित कलोरिमेट्रिक एसेस का उपयोग करते हुए, एप्टामर-AFB₁ परिसंयोजनों के डिससोसिएशन स्थिरांक (K_d) निर्धारित किए गए, जो AFB₁ के लिए नैनोमोलर सांद्रता पर भी मजबूत बाइंडिंग एफ़िनिटी को दर्शाते हैं। सबसे कम K_d मान वाले शीर्ष चार एप्टामर्स CPMA1, L1, L2, और L3 को आगे की विश्लेषण के लिए चुना गया। सर्कुलर डाइक्रोइज़म (CD) स्पेक्ट्रोपोलारिमीट्री ने AFB₁ बाइंडिंग पर एप्टामर्स में संरचनात्मक परिवर्तन दिखाए, जो स्थिर एप्टामर-AFB₁ परिसंयोजन गठन को संकेतित करते हैं। फ्लोरेसेंस-आधारित एसेस, जिसमें SyBr Green-I लेबल-रहित दृष्टिकोण और ग्रेफीन

ऑक्साइड (GO) और FAM-लेबल किए गए एप्टामर ऐसे शामिल हैं, ने इन एप्टामर्स की मजबूत बाइंडिंग काइनेटिक्स की पुष्टि की। AuNPs-आधारित ऐसे का उपयोग करके विशिष्टता और क्रॉस-रिएक्टिविटी अध्ययन ने गैर-लक्ष्य विश्लेषणों, जैसे AFB₂, AFG₁, AFG₂, AFM₁, Ochratoxin A, Fumonisin B1, और Bovine Serum Albumin (BSA) के लिए न्यूनतम बाइंडिंग को दर्शाया, जो AFB₁ के प्रति उच्च विशिष्टता को प्रदर्शित करता है।

इसके अतिरिक्त, AFB₁ के प्रति ssDNA चयनित एप्टामर्स की बाइंडिंग विशिष्टता और स्थिरता का विश्लेषण करने के लिए संगणनात्मक विधियाँ की गईं। एप्टामर्स की द्वितीयक संरचनाएँ MFold वेबसर्वर का उपयोग करके भविष्यवाणी की गईं, जो विविध फोल्डिंग पैटर्न दर्शाती हैं। तृतीयक संरचनाओं को RNA Composer के साथ मॉडल किया गया और DNA-जैसी विन्यासों में परिष्कृत किया गया। AutoDock उपकरणों के साथ आणविक डॉकिंग ने मजबूत बाइंडिंग एफ़िनिटी का प्रदर्शन किया, जिसमें एप्टामर L1 ने सबसे कम बाइंडिंग ऊर्जा (-5.06 kcal/mol) दिखाई। GROMACS में आणविक डायनामिक्स सिमुलेशन ने 100 ns के भीतर एप्टामर-AFB₁ परिसंयोजनों की स्थिरता और डायनामिक्स को मान्य किया, जिसमें RMSD, RMSF, Rg, SASA, और हाइड्रोजन बांड विश्लेषणों ने संतुलन और इंटरैक्शन की स्थिरता की पुष्टि की।

अंतिम उद्देश्य के तहत, AFB₁ के निर्धारण के लिए एक तीव्र, ऑन-साइट और किफायती लैटरल फ्लो एसे (LFA) विकसित किया गया, जिसमें L2 एप्टामर और पूरक cDNA-थियो-गोल्ड नैनोपार्टिकल्स (cDNA-SH-AuNPs) को कलरिमेट्रिक जांच के रूप में उपयोग किया गया, तथा नियंत्रण रेखा पर पॉली (डायएलिल डाइमिथाइलअमोनियम क्लोराइड) (PDDA) को शामिल किया गया। यह एसे दोहरी-रेखा पहचान प्रारूप का अनुसरण करता है: AFB₁ की अनुपस्थिति में, cDNA-SH-AuNPs बायोटिनिलेटेड एप्टामर से परीक्षण रेखा (टेस्ट लाइन) पर संकरण करते हैं और PDDA नियंत्रण रेखा (कंट्रोल लाइन) पर भी बाइंड होते हैं, जिससे दोनों रेखाओं पर दृश्यमान संकेत उत्पन्न होते हैं। AFB₁ की उपस्थिति में, उसका विशिष्ट रूप से एप्टामर से बंधन एप्टामर-cDNA की पारस्परिक क्रिया को बाधित करता है, जिससे परीक्षण रेखा पर पकड़ नहीं बनती और cDNA-SH-AuNPs प्रवाहित होकर केवल नियंत्रण रेखा पर बाइंड होते हैं, जिसके परिणामस्वरूप केवल एक दृश्य संकेत बनता है। यह डिज़ाइन आंतरिक मान्यता तंत्र प्रदान करता है, जो नमूना प्रवास और अभिकर्मकों की कार्यक्षमता को सुनिश्चित करता है। इस एसे ने उच्च विशिष्टता प्रदर्शित की और अन्य माइक्रोटॉक्सिन्स के प्रति नगण्य क्रॉस-रिएक्टिविटी दिखाई। इसने 0.648 माइक्रोमोलर (μM) की न्यूनतम पहचान सीमा (LOD) और 0.01–0.2 μM (~3–6 ppb) की संवेदनशीलता सीमा प्राप्त की, जो AFB₁ की अत्यल्प सांद्रता पर भी

सफल पहचान को इंगित करता है। स्पाइक किए गए मक्का और मूंगफली के नमूनों में AFB₁ की सफल पहचान ने इस एसे की व्यावहारिक उपयोगिता को सिद्ध किया, जिससे यह खाद्य और चारा वस्तुओं में AFB₁ संदूषण की निगरानी के लिए एक संवेदनशील, विश्वसनीय और क्षेत्रीय स्तर पर उपयोग योग्य उपकरण के रूप में स्थापित हुआ।

कुल मिलाकर, इस अध्ययन ने AuNP-आधारित SELEX, अगले पीढ़ी के अनुक्रमण और संगणनात्मक विश्लेषण के संयोजन का उपयोग करके AFB₁ के पता लगाने के लिए उच्च विशिष्टता और संवेदनशीलता वाले DNA-आधारित एप्टामर्स को सफलतापूर्वक विकसित किया। परिणामस्वरूप, इन एप्टामर्स का उपयोग करके एक त्वरित और लागत-कुशल LFA बनाई गई, जिसने वास्तविक स्पाइक किए गए नमूनों में AFB₁ का पता लगाने में मजबूत प्रदर्शन दिखाया, जिससे खाद्य और चारा सामग्रियों में अफ्लाटॉक्सिन संदूषण की निगरानी के लिए एक व्यावहारिक समाधान प्रदान किया।

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Abbreviations

AFB ₁	Aflatoxin B ₁
AFB ₂	Aflatoxin B ₂
AFG ₁	Aflatoxin G ₁
AFG ₂	Aflatoxin G ₂
AFM ₁	Aflatoxin M ₁
AFM ₂	Aflatoxin M ₂
OTA	Ochratoxin A
FB1	Fumonisin B1
BSA	Bovine serum albumin
HCC	Hepatocellular carcinoma
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
AuNPs	Gold nanoparticles
LFAs	Lateral flow assays
SG-I	SyBr Green-I
GO	Graphene oxide
K _d	Dissociation constant
NGS	Next generation sequencing
MEME	Multiple Em for Motif Elicitation
MAST	Motif Alignment and Search Tool
µg	Microgram
ml	Microliter
Kcal	Kilocalorie
KJ	Kilo joule
M	Molar
mM	Mili Molar
µM	Micro Molar
nM	Nano Molar
TLC	Thin Layer Chromatography
GC	Gas Chromatography

IR	Infrared
HPLC	High performance liquid chromatography
HR-LCMS	High resolution liquid chromatography-mass spectrometry
RIA	Radioimmuno assay
ELISA	Enzyme linked immunosorbent assay
CD	Circular dichroism
BE	Binding energy
MD	Molecular dynamic
SDF	Structure Data File
PDB	Protein databank file
2D	2 dimension
3D	3 dimension
ns	nano second
nm	nano meter
NVT	Constant Number of atoms, Volume, and Temperature
NPT	Constant Number of atoms, Pressure, and Temperature
VWE	Van der Waals energy
EE	Electrostatic energy
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
Rg	Radius of gyration
SASA	Solvent accessible surface area