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Journal Pre-proof

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Author contributions

Maryam Mousivand: Conceptualization, Methodology, Validation, Software, Formal Analysis, Investigation, Writing – Original Draft, Review & Edition;

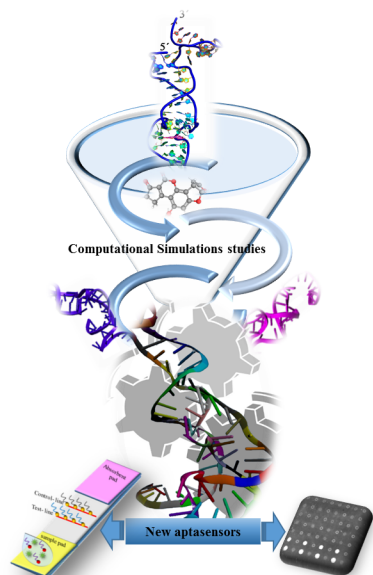
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1 **High performance aptasensing platform development through in silico aptamer**
2 **engineering for aflatoxin B1 monitoring**

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13
14 **Abstract**

15 Due to the technical challenges of small binding aptamer development, reliable
16 computational simulation studies can be considered as effective tools to design novel and
17 high functional mycotoxin aptameric probes. Here, two novel aflatoxin B1(AFB1) binding
18 aptamers were successfully exploited as recognition elements in the lateral flow aptasensors
19 and the reflective phantom interface (RPI) platform. Using the parent aptamer previously
20 designed through genetic algorithm based in silico maturation (ISM) strategy, F20, a new
21 variant, F20-T, was obtained here via coupling truncating strategy and computational
22 simulation approaches. Two aptamer-gold nanoparticle strip biosensors were developed
23 based on the designed probes for the simple and rapid detection of AFB1 in competitive
24 format. The F20-based strip was more sensitive than that exploiting the truncated aptamer,
25 with limits of detection (LOD) of 0.1 and 0.5 ng/mL, respectively. Based on the in silico and
26 experimental selectivity evaluations of both test strips towards other mycotoxins, including
27 aflatoxin B₂, M₁, G₁, G₂, Ochratoxin A and Zearalenone, F20-T based test strip revealed
28 higher selectivity for AFB1. Both developed aptasensors successfully detected AFB1 in
29 maize flour within 30 min using a simple strip reader. Exploiting of F20 and F20-T aptamers
30 in an exclusive technology called RPI platform led to successful AFB1 detection, as well.
31 Both designed aptameric probes can be regarded as potential recognition elements to develop
32 screening tools for rapid, low cost and on-site AFB1 detection. Our findings highlighted the

33 reliable and robust application of computational simulation studies for novel small binding
34 aptamer development and consequently open up a much-needed avenue to design various
35 aptasensing platforms in green and cost effective ways.

36 **Key words:** aptasensor, computational simulations, lateral flow, aflatoxin B1, small
37 molecule

38

39 **Introduction**

40 Among aflatoxin contaminations, AFB1 is the most prevalent and identified as the first
41 hazard class by the International Agency for Research on Cancer (IARC, 2000). To monitor
42 the low permissible concentrations of AFB1 in complex food and feed matrices (EC, 2010),
43 various analytical methods have been developed. In spite of high sensitivity and specificity of
44 chromatography based instrumental techniques, high cost, time-consuming and requiring
45 highly skilled personnel are the main obstacles for their large-scale applications (Miklos et
46 al., 2020). Regarding to the rapidity and simplicity, antibody-based immunoassays are widely
47 used in routine food analysis. Considered as non- immunogenic compounds, the antibody
48 generation for mycotoxins has several issues in terms of high cost, time consume and
49 laborious (López-Puertollano et al., 2018).

50 Responding to increasing demand for affordable, accurate and simple devices for mycotoxin
51 detection especially outside the laboratory, various biosensing platforms have been developed
52 as alternative analysis tools to ensure food safety (Chauhan et al., 2016). While antibodies
53 have become the most popular recognition elements in biosensor words for four decades (Di
54 Nardo et al., 2021), aptamers have emerged as a potent rival of antibodies owing to inherent
55 advantages over them .Unlike antibodies, aptamers can be selected over various targets
56 regardless of their immunogenicity through an in vitro selection process called systematic
57 evolution of ligands by exponential enrichment (SELEX). The low cost, ease of synthesis,
58 prolonged shelf-life and regeneration under a broad range of conditions make aptamers as
59 attractive candidates to incorporate in mycotoxin biosensing devices (Yang et al., 2013).

60 Despite the increasing demand for mycotoxin binding aptamers, a set of technical challenges
61 are still the main bottlenecks for their research and commercialization. The SELEX process,
62 known as a gold-standard methodology for aptamer development, is still cost, laborious and
63 time-consuming. Also, the limitation of initial library diversities and sequence bias during

64 iterative PCR reduced the success rate of SELEX by 50% for recovering high binding affinity
65 aptamers (Sun and Zu, 2015). These drawbacks are more highlighted in the case of small
66 molecules binding aptamers because most affinity binding assays are not sensitive enough to
67 separate small target-bound sequences from other ones due to drastic different size between
68 small molecules such as mycotoxins and their binding aptamers (Ruscito and DeRosa, 2016).
69 Also, the limited functional groups in small molecules decrease probability of finding high
70 functional aptamers that can interact with the target via electrostatic, H-bonds, hydrophilic or
71 π - π -stacking interactions (Mascini, 2009).

72 Despite the requirement of high affinity and novel aptamers to design mycotoxin biosensing
73 platforms, a few aptamers have been developed for the class of hazardous compounds
74 (McKeague et al., 2015). Duo to the very few novel aptamers for mycotoxins, coupling them
75 to the various transducer systems can be considered as a compensatory solution to design
76 high performance mycotoxin biosensing devices (Yang et al., 2013). Among various
77 aptasensing platforms, lateral flow based aptasensor development can be considered as a
78 promising answer to the increasing demand for simple, low cost, portable and on site
79 detection of AFB1. However, until now a few lateral flow based aptasensors have been
80 developed for AFB1 monitoring (Shim et al., 2014, Zhu et al., 2017, Zhang et al., 2018b,
81 Zhao et al., 2020), all of which have exploited Apt1 aptamer patented by Neoventures
82 Biotechnology Inc. (NeoVentures Biotechnology Inc.). Exploiting the only AFB1 aptamer
83 (Apt1) in almost all developed aptasensors (Jia et al., 2019) makes it necessary to design
84 novel aptamers as new recognition elements for existing biosensing platforms.

85 Being as main components, the bioreceptor and transducer properties have critical effects on
86 the biosensor sensitivity and selectivity. Recently, various modified enzyme, recombinant
87 antibody fragments and nanobodies have been developed through bioengineering techniques
88 to increased acceptance and commercialization of the biosensing platforms (Hock et al.,
89 2002). However, the technical barriers of aptamer development especially for small
90 molecules have constituted major bottlenecks for aptamer engineering research and high
91 functional aptamer discovery (Crivianu-Gaita and Thompson, 2016).

92 To overcome the challenges, experimental findings can be combined to the in silico
93 approaches to refine the affinity and specificity of mycotoxin binding aptamers. Recently,
94 aptamer engineering with the aim of sequence or scaffold optimizations have gain attentions
95 as a promising area of active research to design and discover the novel mycotoxin binding

96 aptamers (Mousivand et al., 2020; Ciriaco et al., 2020; Hasegawa et al., 2016). In the case of
97 small molecule targets, sequence truncating strategy can be considered as an effective
98 approach to improve aptamer affinity and specificity via different size reduction between
99 them and their aptameric partners (Aissa et al., 2020). Due to the cost and time constraints,
100 the experimental evaluation of all designed aptamers can be considered as an important
101 limitation for small molecules binding aptamers developing. Reliable computational
102 simulations have the capacity to virtually screen a large database of aptamers and clarify their
103 binding modes in cost and time effective ways (Zhang et al., 2018a; Mousivand et al., 2021;
104 Chushak and Stone, 2009).

105 The aim of our study was to design new aptasensing platforms via *in silico* engineered
106 aptamers instead of exploiting the same aptameric probe in different transducer systems.
107 Therefore, F20 aptamer, previously designed based on Apt1 sequence through genetic
108 algorithm based ISM approach (Mousivand et al., 2020, 2021), has been applied to develop a
109 new truncated AFB1 aptamers, F20-T, via coupling truncating strategy and computational
110 simulations. Both new designed AFB1 binding aptamers were exploited as new recognition
111 elements in nanogold-based lateral flow aptasensors and RPI platform for AFB1 detecting.

112 **2. Material and Methods**

113 **2.1. Computational Studies**

114 **2.1.1. Aptameric probes**

115 The main aptameric probe, F20, was previously designed based on the Apt1 sequence
116 (Patent: PCT/CA2010/001292) subjected to generate the second probe, F20-T, through
117 coupling truncating strategy and computational studies as follow.

118 The K_d values of F20, F20-T and Apt1 were estimated through unmodified AuNPs-based
119 colorimetric assay (details in the Supporting Information) according to Mousivand et
120 al.(2020).

121 **2.1.2. Library generation and secondary structure analysis**

122 Based on F20 sequence, different variants were constructed using truncating strategy. The
123 created library contained oligonucleotides variable in length, randomly truncated at either the
124 5', 3' or both end of sequences. The secondary structures of potent aptamers in the truncated

125 library were predicted using the Mfold web server at 37°C and at ionic concentration of 1 M
126 of Na⁺, 0 M of Mg²⁺ based on the free energy minimization algorithm (Zuker, 2003).

127 **2.1.3. Molecular docking technique**

128 Virtual screening of the truncated library was performed using AutoDockTools (ADT) 1.5.4
129 package (Morris et al., 2009) to evaluate AFB1 binding affinity of individual aptamers. The
130 crystal structures of AFB1 were obtained from the PubChem database and considered as a
131 flexible ligand while the predicted 3D-structure of aptamers were kept as rigid receptors.
132 Three dimensional modeling of truncated ssDNA aptamer was constructed through a
133 sequentially pipeline according to Mousivand et al. (Mousivand et al., 2020). Accordingly,
134 different complexes were ranked based on the obtained docking scores including binding
135 energy, type of favorable interactions and the binding sites. The best aptamer was then
136 compared to the parent aptamer, F20, in terms of selectivity over different mycotoxins
137 including AFB1, AFB2, AFG1, AFG2, AFM1, ZEN and OTA using molecular docking
138 technique. The selected aptameric probe was further evaluated with the aid of molecular
139 dynamic simulations.

140 **2.1.4. Molecular dynamic simulations (MDs)**

141 The conformational changes and binding mode of F20 and the corresponding truncated form,
142 F20-T, were simulated alone in water and in complex with AFB1 during 50 ns of MD
143 stimulations. All simulations were conducted using GROMACS 5.1.4 software package
144 (Berendsen et al., 1995) under AMBER99SB force field (Perez et al., 2007). SwissParm web
145 server (Zoete et al., 2011) was employed to generate the ligands topology and parameter files.
146 The best ranked complex taken from docking results was immersed in the center of a
147 dodecahedron periodic box containing TIP3P water model with 1 nm away from each wall.
148 After the MDs settings according to Mousivand et al., (2021), the system went through a final
149 50 ns MD simulations at constant pressure and temperature conditions and the coordinates of
150 the complexes were recorded every 10 ps for the subsequent analysis. MD simulations were
151 analyzed using GROMACS tools and all visualizations were performed via Discovery Studio
152 v3.5 (Biovia, 2015), VMD (Humphrey, 1996) and PyMOL (De Lano, 2002) softwares.

153 **2.1.5. Binding free energy MM-PBSA calculation**

154 To estimate the binding affinity over AFB1, the molecular dynamic trajectory files of F20
155 and F20-T complexes were subjected to MM-PBSA analysis using g_mmpbsa tool (Kumari

156 et al., 2014). Gibbs free energy and its different components including electrostatic, van der
157 Waals, polar solvation and non-polar solvation energies were estimated. Regarding to the
158 interest in relative binding of the selected aptamers, the entropy was not calculated. The most
159 important nucleotides involved in binding affinity toward AFB1 were retrieved through
160 energy decomposition per residue as well.

161

162 **2.2. Experimental studies**

163 **2.2.1. Reagents and Apparatus**

164 Gold (III) chloride trihydrate (ACS reagent), bovine serum albumin (BSA), Tris-(2-
165 carboxyethyl) phosphine hydrochloride (TCEP), and mycotoxin standard solutions were
166 purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained
167 from VWR International (Milano, Italy). 96-Microwell transparent plates were purchased
168 from Nunc (Roskilde, Denmark). Thiol-modified aptamers and biotin modified probes were
169 synthesized by TAG Copenhagen A/S (Denmark) (Supplementary Information, Table S1).
170 The cellulose fiber pads (sample and absorbent pads) and nitrocellulose membranes (HF180
171 plus card, 60mm × 300 mm) were obtained from Millipore (Billerica, MA, USA). Test and
172 control lines were loaded on the nitrocellulose membrane by means of an XYZ3050 platform
173 (BioDot, Irvine, CA, USA), equipped with two BioJet QuantiTM 3000 Line Dispenser for
174 noncontact dispensing. The membrane was cut into 4.6 mm test strips by a CM4000
175 guillotine (BioDot, Irvine CA, USA). The color intensity of test and control lines were
176 *scanned and then analyzed by QuantiScan 3.0 software (Biosoft, Cambridge, UK). The ultra-*
177 *pure water used throughout all experiments obtained by a Milli-Q system at 18.2 MΩ.*

178

179 **2.2.2. Preparation of gold nanoparticles (AuNPs)**

180 The synthesis of ~ 30-nm-diameter gold nanoparticles was performed through the HAuCl₄
181 reduction with sodium citrate (Cavalera et al., 2020). Typically, 1 mL of 1% sodium citrate
182 was added to 100 mL of boiling 0.01% tetrachloroauric acid under constant stirring and
183 heating. When the suspension color changed from light yellow to deep red, heating was
184 continued for another 10 min and then cooled to room temperature. The prepared gold
185 nanoparticle size was confirmed through displaying a λ_{\max} equal to 525 nm by UV-Visible
186 spectrometry. The AuNPs solution was adjusted to pH 8.5 and concentrated to 10X at 14000
187 rpm for 10 min and then stored at 4°C for subsequent conjugation.

188

189 **2.2.3. Preparation of gold nanoparticles-aptamer conjugates (AuNPs-Apt)**

190 To activate the modified aptamers, 2 μL of 0.125 mM acetate buffer (pH 5.2) and 1.5 μL of
191 freshly prepared 10 mM TCEP were added to 10 μL of 100 mM thiolated aptamers and
192 incubated for 1h at room temperature. Subsequently, 500 μL of concentrated gold
193 nanoparticles was transferred to the TCEP-treated aptamers and left reacting for at least 16 h
194 in the dark at 4°C. After adding 10 μL of 0.25 mM tris acetate buffer (pH 8.2), the suspension
195 was aged through adding 50 μL of 1 M NaCl dropwise (5 μL every 20 min) and left for
196 another over night at 4°C. The prepared suspension was centrifuged at 10000 rpm for 10 min
197 at 4°C and then resuspended in 200 μL of 25 mM tris acetate buffer (pH 8.2) containing 100
198 mM NaCl. After centrifugation at the same condition, the pellet was resuspended in 500 μL
199 of 25 mM tris acetate buffer (pH 8.2) containing 300 mM NaCl and stored at 4°C (Liu and
200 Lu, 2006).

201 With the aim of increasing conjugate stability, different final concentrations of aptamer F20
202 and F20-T (0.2, 0.5, 1, 2 and 3 μM), incubation time (4 and 24 h) and two conjugate
203 preservation buffer composition including (i) tris acetate buffer (25 mM, pH 8.2) containing
204 300 mM NaCl and (ii) borate buffer (20 mM, pH 8) containing 1% BSA, 2% sucrose, 0.25%
205 Tween 20 and 0.02% NaN_3 were further investigated.

206 **2.2.4. Preparation of the test strip**

207 The biotinylated DNA probes 1 and 2 were immobilized on the nitrocellulose membrane to
208 form test and control lines, respectively, at a distance of 4 mm from each other. Prior to
209 loading, 50 μL of the biotinylated DNA probe (100 μM) was mixed with 250 μL of 2 mg/mL
210 streptavidin in PBS buffer (0.01 M, pH 7.4). After incubating the suspension at 4°C for 1h,
211 700 μL of PBS buffer (0.01 M, pH 7.4) was added. The membrane was kept at room
212 temperature for 5 min and dried at 37 °C under vacuum for 45 min. The sample and absorbent
213 pads were pasted on the bottom and top of the nitrocellulose membrane respectively with 1–2
214 mm of overlap and the prepared master card was cut into 4.6 mm test strips.

215 The minimum required DNA probes 1 and 2 were evaluated through developing red spots on
216 the test and control zone respectively as a function of the hybridization reaction between 1
217 μM of AuNPs-Apt (F20 or F20-T) and various concentrations of both DNA probes (5, 15, 30,
218 and 60 μM). After initial optimization, four nitrocellulose membranes with different
219 concentrations of DNA probes 1 and 2 (100 nM, 500 nM, 2.5 μM and 5 μM) were prepared

220 and the color intensity at the test and control lines were further evaluated in the presence of
221 various concentrations of AuNPs-Apt conjugates. Different concentrations of both
222 conjugates, F20 (1 μM , 0.2 μM and 0.1 μM) and F20-T (2 μM , 0.4 μM , and 0.2 μM), were
223 determined based on the obtained results from AuNPs-Apt preparation.

224 **2.2.5. Aptamer based lateral flow assay procedure**

225 The performance of two designed lateral flow aptasensors for AFB1 detection were
226 evaluated. Therefore, 20 μL of various concentrations (0-50 ng/mL) of AFB1 standard
227 solution in methanol were mixed with 20 μL of AuNPs-Apt conjugate (at optimized
228 concentration) in microplate wells for 10 min at room temperature. After adding 20 μL of
229 PBS buffer (0.01 M, pH 7.4) and 20 μL 10% Tween 20, the test strips were placed into the
230 wells and the color intensity of the lines was analyzed 20 minutes later.

231 The measured area of the test and control line ratio versus AFB1 concentration in three
232 replicates was plotted to obtain a calibration curve. The IC_{50} value was calculated by AAT
233 Bioquest program using a four parameter logistic regression model (AAT Bioquest, Inc.,
234 Sunnyvale, CA). The limit of detection (LOD) was defined as the lowest concentration which
235 corresponded to the T/C of the blank minus three standard deviations of the blank.

236

237 **2.2.6. Selectivity of lateral flow test strip**

238 The designed test strips were evaluated and compared in terms of selectivity toward AFB1
239 and cross reactivity with other mycotoxins. Under the optimal conditions, the selectivity of
240 F20 and F20-T conjugates were determined over various mycotoxins including AFB₂, AFG₁,
241 AFG₂, AFM₁, ochratoxin A and zearalenone at the concentration of 10 ng/mL in three
242 replicates. The ratio of the T/C for each mycotoxin was calculated, normalized according to
243 the AFB1 result and then expressed as selectivity percentage. The obtained mean for each
244 mycotoxin was compared between two conjugates through the independent samples t-test
245 using SPSS v.16.0; ($P \leq 0.05$).

246

247 **2.2.7. Test strip performance under methanol content**

248 Due to the mycotoxin extraction using conventional organic solvents, the methanol
249 interference on the DNA hybridization process occurring in the nitrocellulose membrane was
250 studied. Therefore, 20 μL of AFB1 standard solution (10ng/mL) diluted by various
251 concentrations of aqueous methanol (5, 10, 25, 35, 50% v/v) was mixed with 20 μL of F20 or

252 F20-T AuNPs-Apt conjugates in microplate wells for 10 min at room temperature. The color
253 intensities developed at the test and control lines were scanned 20 min later and quantified.

254

255 **2.2.8. Sample assay procedures**

256 The reliability and accuracy of both designed lateral flow test strips were conducted on four
257 reference materials of maize flour with HPLC certified concentrations of AFB1 (<LOD, 5,
258 11.3 and 28.9 ppb), friendly obtained from Turin University. To perform the extraction
259 process, one gram of each flour sample was extracted with 5 mL of 70% aqueous methanol
260 through 2 min vortexing. After 15 min of settling, 20 μ L of the supernatant was applied as the
261 sample and mixed with 20 μ L of AuNPs-Apt conjugate in a microplate well for 10 min at
262 room temperature. To optimize the performance of the competitive format, two distinct
263 membranes loaded with 2.5 and 5 μ M of DNA probes were exploited. For further
264 improvement, the incubation time of the strip in the well increased and the developed color
265 was scanned after 20 and 30 min of reaction, as well. To evaluate the matrix effects and
266 possibility of obtaining false positive results, the T/C ratios of the samples 1 for both
267 designed test strips were compared to the blank samples in their corresponding calibration
268 curves. The calculated T/C ratios for other samples were normalized based on sample 1 value
269 as well.

270

271 **2.2.9. Aptamer Binding affinity evaluation over AFB1 via RPI technology**

272 Aptamer F20 and its truncated form, F20-T, were applied as recognition elements in RPI
273 platform for AFB1 detecting. To estimate the K_d values, 400 μ l of the aptameric probes (10
274 μ M) were immobilized on the microarray surface and the binding affinity was estimated in
275 the presence of various concentration of AFB1-BSA conjugate (0.01, 0.07, 0.28, 1.09 and
276 4.20 μ g/mL) at the fixed times. The binding affinity of F20 and F20-T aptamers toward
277 AFB1 were simultaneously compared to the several aptameric probes including C52, C52T,
278 G12 and H1 designed through previous study (Mousivand et al., 2020) and two antibodies
279 under the same condition as well.

280 **3. Results and Discussion**

281 **3.1. Truncated library construction and thermodynamic analysis**

282 Considering the technical challenges for small molecule aptamer development, the truncating
283 strategy was employed to design new truncated aptameric probes. The truncated library

284 containing 19 potent aptamers with various lengths from 10 to 40 bp generated based on the
285 parent sequence truncation. The minimum free energy of secondary structure formation (ΔG)
286 in the truncated library was in the range -8.01 to 1.88 Kcal/mol (Supplementary Information,
287 Table S2). The secondary structure prediction revealed that most of the designed sequences in
288 the library had simple hairpin loop (H-loop) structures except those of F20-30 and F20-40
289 that displayed internal loop and multibranch loop, respectively.

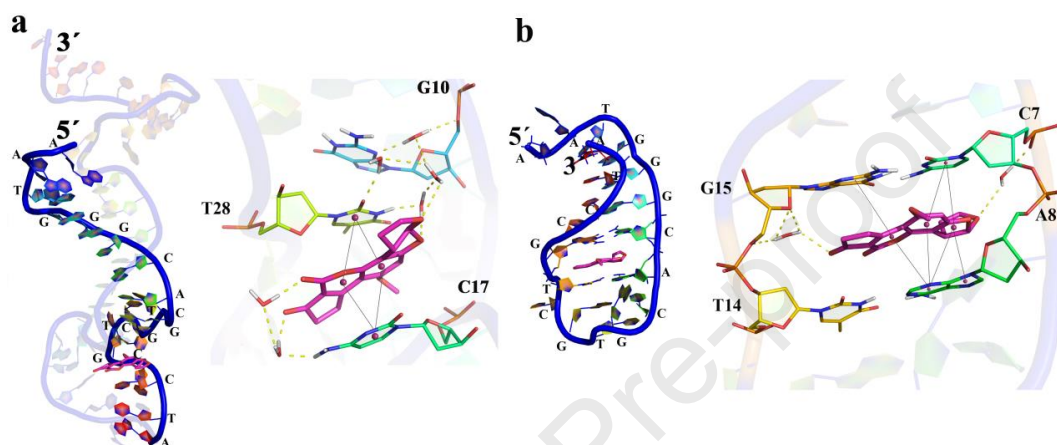
290 **3.2. Virtual screening of AFB1 binding aptamers**

291 To predict the binding energy and critical interacting residues, AFB1 was docked over the
292 aptamers in the truncated library and the estimated docking scores were in the range 1.66 to
293 4.17. Compared to other truncated aptamers, F20-T with H-loop structure and 19 bp in length
294 showed the highest binding affinity towards AFB1. F20-T binding pocket includes C7, A8,
295 G15, G10 and T14 residues that interact AFB1 coumarin and carbonyl groups through
296 hydrogen bond formation and hydrophobic interactions (Supplementary Information, Fig S1;
297 Tables S2 & S3). Although F20-T and its parent, F20, had the same secondary structure but
298 their modes of interaction with AFB1 were different and determined as intercalation and
299 minor groove binding, respectively. Compared to the parent sequence, the selectivity of the
300 truncated aptamer had been increased over all the evaluated mycotoxins except for ZEA
301 (Supplementary Information, Table S4). Depending on the conformational changes and
302 losing probable binding sites, the truncating strategy can lead to increase or decrease binding
303 affinity and selectivity of designed aptameric probes.

304 **3.3. Molecular dynamic simulations**

305 To clarify the truncation effects on the complex structural stability, a 50 ns molecular
306 dynamic study was conducted on F20-T –AFB1 complex and the results were compared with
307 MDs studies of the parent sequence, F20. The system convergence during MDs timescale was
308 confirmed via insignificant changes in potential energy (Panman et al., 2017). The
309 conformational changes and binding interactions of F20-T and F20 –AFB1 complex were
310 evaluated with respect to the lone aptamer during 50ns of MDs. Regarding to the flexible
311 nature of nucleic acids, all trajectory analysis were performed for both all atoms aptamer and
312 its binding pockets as suggested by other study (Sharma et al., 2009). Similar to F20 aptamer,
313 the structural stability of F20-T increased after interacting with AFB1 and the corresponding
314 Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) values
315 revealed a decreasing trend compared to the lone aptamers during MDs (Supplementary

316 Information, Table S5&S6 Fig. S2&S3). Visualization of trajectory files showed that AFB1
 317 inserted between consecutive base pairs in the stem region of F20-T and subsequently the
 318 radius of gyration (Rg) values increased for all atoms and binding pockets (Supplementary
 319 Information, Fig S4). While AFB1 recognized F20 aptamer through minor groove edges of
 320 C17 and T28 residues and interacted with the binding pocket as a pseudo base subsequently
 321 leading to increase compactness and structural stability along with reduction in Rg value (Fig.
 322 1).



323
 324 Figure 1. The molecular dynamic simulation results of F20 (a) and F20-T (b) - aflatoxin B1 complexes and
 325 residues involved in binding interaction in 3D representation. Yellow dash lines represent conventional
 326 hydrogen bonds, purple dots and the lines represent electrostatic and hydrophobic interactions, respectively. The
 327 truncated segment of the parent aptamer (a) has been highlighted in bold blue color.

328 The hydrogen bond formation between the aptameric probes and AFB1 along with water
 329 intermediate interactions was evaluated to determine their role in the complex stability during
 330 MDs. The average H-bonds monitored between truncated aptamer and AFB1 was
 331 approximately 7 times higher than that of F20-AFB1 complex and estimated as 0.28 ± 0.59 .
 332 Although both aptamers mainly interacted with AFB1 through dynamic hydrogen bonds, H-
 333 bonds formed with F20-T seem to be more stable. According to the hydrogen bond
 334 occupancy percentage ≥ 10 ns, the only stable interaction explored in the binding pocket of
 335 the truncated aptamer with residue G15 was estimated as 15.5% (Supplementary Information,
 336 Table S7). The dynamic H-bonds between the surrounding water molecules with the binding
 337 pocket and AFB1 in F20-T complex as well as F20 complex play a key role in the structural
 338 integrity through hydrating of DNA and ligand (Supplementary Information, Table S8) as
 339 suggested by other study (Dolenc et al., 2005).

340 3.4. MM-PBSA calculations

341 The obtained MDs trajectories of F20/F20-T and AFB1 complexes were analyzed to estimate
 342 their free binding energies and different components. The binding affinity of AFB1 for the
 343 truncated aptamer is reduced by half compared to the parent sequence but it was equivalent to
 344 that of Apt1 aptamer (Mousivand et al., 2021) and estimated as -47.44 KJ/mol. According to
 345 the free binding energy component inspection, Van der Waals, non-polar and electrostatic
 346 interactions showed the major contributions to complex stability in both aptameric probes,
 347 respectively. The negative effect of polar interactions in free solvation energy can be
 348 attributed to the hydrophobic nature of AFB1 (Table 1). This finding was in line with other
 349 studies that confirmed the destabilizing role of polar interactions in binding affinity over
 350 AFB1(Mousivand et al., 2021; Almedia et al., 2018). In concordance with the docking
 351 studies, per-residue energy decomposition analysis revealed that residues C7, A8, C9, G10,
 352 T14, and G15 are the key interacting nucleotides in F20-T binding pocket over AFB1,
 353 respectively (Supplementary Information, Fig. S5). Also, the high consistency between
 354 experimentally determined binding affinity (K_a) values of F20 (3.55×10^{-5} nM), Apt1 ($1.30 \times$
 355 10^{-5} nM) and F20-T (1.12×10^{-5} nM) over AFB1 with their free binding energies estimated as
 356 -70.04, -48.67, and -47.44 KJ/mol highlighted the in silico approaches as promising tools for
 357 functional aptamer designing.

358 **Table 1.** Comparison of the free binding energy components for the aptamer-AFB₁ complexes obtained from
 359 MM-PBSA method given in KJ/mol.

aptamers	ΔE_{vdw}	ΔE_{elec}	ΔG_{polar}	$\Delta G_{non-polar}$	$\Delta G_{binding}$
F20-T	-40.44±3.67	-8.90±5.50	20.44±30.87	-15.58±5.66	-44.47
F20	-37.37±4.04	-17.13±10.92	-2.78±34.66	-12.75±1.42	-70.04
Apt1	-48.34 ± 3.67	-11.90 ± 5.50	23.44±30.87	-11.88±5.66	-48.67

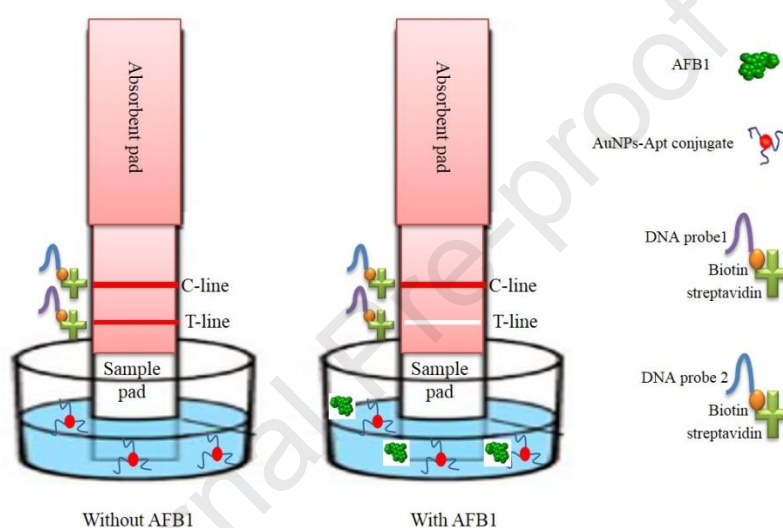
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361 3.5. Development of the aptamer-based lateral flow test strip

362 The principle of the designed test strip was relied on the competition between the DNA probe
 363 1 immobilized on the test line and AFB1 to react with AuNPs-aptamer conjugate in the
 364 sample as shown in Scheme 1. As it was expected for a competitive format, the color
 365 intensity of the test line was inversely proportional to AFB1 concentration in the samples.
 366 Regardless the presence or absence of AFB1, the excess AuNPs-aptamer conjugates were
 367 captured through the linker complementary DNA probe 2 in the control line to valid the
 368 detection process and normalize strip-to-strip variation. In spite of conventional use of BSA

369 in the lateral flow test strips to block the non specific binding sites (Molinelli et al., 2008; Xu
 370 et al., 2010), the high binding affinity of BSA over AuNPs-aptamer conjugates and
 371 hybridization interference in the test and control lines hindered the treatment of nitrocellulose
 372 membranes with BSA. This finding was in line with results of other research that reported
 373 that the BSA binds the citrate-stabilized gold nanospheres through an electrostatic attraction
 374 via the lysine residues (Brewer et al., 2005) or by a thiol ligand exchange reaction with the
 375 unpaired cysteine residue (Tsai et al., 2011).

376

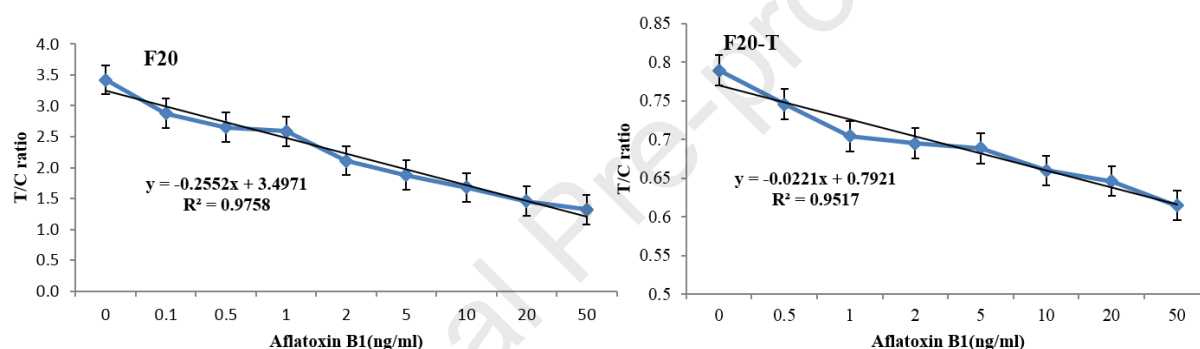


377

378 Scheme 1. Schematic illustration of aptamer-based lateral flow strip for aflatoxin B1 (AFB₁) detection and result
 379 interpretation in the presence or absence of AFB₁.

380 Difficulty in standardizing the amount of AuNPs-aptamer conjugates caused strip-to-strip
 381 variation even at a constant target concentration. To overcome this problem, the conjugates
 382 were mixed with the sample before performing the test instead of pre-adsorbing on the
 383 conjugated pads (Molinelli et al., 2008). Also, normalization can be achieved via some data
 384 corrections through using the control line intensity due to its association with variability of
 385 the gold conjugate amount and any other factors affecting the detection procedure. Therefore,
 386 the color intensity of the control line was exploited to normalize the result variations by
 387 dividing the test line area (T) by the control line area (C) (Anfossi et al., 2010). To obtain the
 388 normalized standard curve, the optimized concentration of AuNPs-aptamer conjugates was
 389 mixed with different AFB₁ concentrations before the detection process on the test strip and
 390 then the T/C ratio was measured. According to the obtained results, the designed lateral flow
 391 based on F20 and F20-T aptamers showed IC₅₀ of 2.9 and 15.4 ng/mL, and a dynamic range

392 of 0.1-50 and 0.5 -50 ng/mL, respectively. Based on the estimated LOD, the parent aptamer
 393 (0.1 ng/mL) was more sensitive compared to its truncated form (0.5 ng/mL) and showed
 394 wider T/C ratios over different AFB₁ concentrations as well (Fig. 2). The better performance
 395 of F20 based strip can be attributed to the longer length of the parent aptamer, which provides
 396 higher gold surface coverage and more stable conjugate formation. Compared to Apt1 based
 397 lateral flow strip with a quantitative LOD of 1.05 ppb (Zhang et al., 2018b), both designed
 398 lateral flow strips were able to detect AFB₁ more sensitively and accurately. The high
 399 consistency between the experiments and in silico findings highlighted the reliability of the
 400 computational simulation techniques in the search of functional aptamers to be exploited for
 401 biosensor development.



402

403 Figure 2. Calibration curves obtained by the normalized T/C ratio versus the AFB₁ concentration (ng/mL) for
 404 F20-T and F20 lateral flow strips.

405 3.6. Optimization of the test strip components

406 3.6.1. Aptameric probe modifications

407 The load capacity of the thiol modified oligonucleotides onto the surface of Au nanoparticles
 408 can be increased due to the well-known chemical interaction between alkyl thiol and gold
 409 through Au-S bond (Love et al., 2005). Therefore, both aptameric probes had been tagged
 410 with a thiol group at the 3' during the synthesis process. In order to reduce the adsorption of
 411 the main sequence on the surface of Au-NPs and interference with the binding interaction, a
 412 poly adenine (A) spacer region between the thiol group and the aptameric sequences was
 413 designed. According to other studies, using the oligonucleotide spacer improves the
 414 hybridization efficiency and its composition and length influenced on the Au surface
 415 coverage with the functionalized oligonucleotides (Hurst et al., 2006).

416 **3.6.2. Gold Nanoparticles-Aptamer Conjugates (AuNPs-Apt)**

417 Despite the promoting role of NaCl in the Au/thiol interactions, it induces the gold
418 nanoparticles aggregation during the AuNPs-Apt conjugate preparation and coloring shift
419 from red to purple as well. Due to the protecting from salt-induced aggregation through
420 ssDNA loading on the NPs surface (Hurst et al., 2006; Wu et al., 2018), the optimum final
421 concentrations of F20 and F20-T based conjugates were estimated as 1 and 2 μM respectively
422 (Supplementary Information, Fig. S6). Requiring lower parent aptamer concentration for
423 stable conjugate preparation may be attributed to its better gold surface coverage. Also, the
424 longer incubation time of NPs with both TCEP-treated thiol aptamers under different
425 concentrations led to the more stable conjugate preparation. Due to the prevention of NPs
426 aggregates, tris acetate buffer containing NaCl was identified as a better conjugate
427 preservation buffer. Despite common use of BSA for stabilizing gold colloids conjugated to
428 antibodies (Molinelli et al., 2008; Xu et al., 2010), the high binding affinity between citrate-
429 stabilized gold nanospheres and BSA (Tsai et al., 2011) caused NPs aggregation during
430 AuNPs-Apt conjugate preservation.

431 **3.6.3. The test and control lines optimization**

432 Regarding to the electrostatic adsorption of streptavidin on the nitrocellulose membrane and
433 its high binding affinity to biotin, the biotinylated DNA probe-streptavidin conjugates were
434 immobilized on the test and control lines. Owing to four identical binding sites of the
435 streptavidin to biotin (Yuan et al., 2010), the ratio between streptavidin and the biotinylated
436 DNA probes were set as 1:4. Under the constant concentration of both AuNPs-Apt conjugates
437 (F20 and F20-T), the red hybridization dots were visualized for all evaluated initial
438 concentrations of the biotinylated DNA probes and then their minimum required
439 concentrations were estimated as 5 μM (Supplementary Information, Fig. S7). Further
440 improvement of the test and control line performance were achieved when F20 and F20-T
441 conjugates at their optimum concentration (0.2 and 0.4 μM , respectively) were hybridized
442 with DNA probes 1 and 2 at the final concentration of 2.5 μM on the membrane
443 (Supplementary Information, Fig. S8).

444 **3.7. Test strip performance under methanol content**

445 The adverse effects of organic solvents on the aptamer/antibody activity, colloidal of
446 AuNPs and the co-extraction of fatty materials reduce the biosensing platforms performance

447 (Anfossi et al., 2010; Molinelli et al., 2009). Therefore, in the presence of a constant
448 concentration of AFB1 (10 ng/mL), the hybridization reactions of both AuNPs-aptamer
449 conjugates with DNA probes on the membrane were investigated under various methanol
450 contents (5 -50 %). According to the results, the color intensities on the test and control lines
451 gradually increased along with increasing methanol content, so that both conjugates showed
452 the highest hybridization percentage at a concentration of methanol corresponding to 50%
453 (Supplementary Information, Fig. S9). In contrast to earlier studies (Shim et al., 2014; Zhou
454 et al., 2016), these findings revealed that the greater methanol content not only did not reduce
455 DNA hybridization but increased its rate and then should be considered as an effective factor
456 on the lateral flow responses especially in the competitive formats. These finding are
457 consistent with those of other studies that found the hybridization rate of DNA-functionalized
458 NPs (Smith and Liu, 2010) and molecular beacon (Dave and Liu, 2010) were significantly
459 faster in most organic solvents compared with water attributed to the reduced activation
460 energy barrier for the hybridization reaction in the presence of organic solvents.

461

462 **3.8. The selectivity of the test strips**

463 Both developed lateral flow strips were evaluated in terms of selectivity toward AFB1 and
464 cross reactivity over AFB2, AFM1, AFG1, AFG2, OTA and ZEA through experimental and
465 in silico methodologies. According to the experimental results, F20 and its truncated form
466 based test strips showed the highest affinity towards AFB1 and a general cross reactivity over
467 other mycotoxins. F20-T based lateral flow assay showed higher selectivity than its parent
468 aptamer based strip toward others mycotoxins, except toward ZEA (Fig. 3). The statistical
469 significant difference between the calculated mean selectivity of F20 and F20-T based test
470 strips for each mycotoxin was confirmed by independent samples t-tests. In agreement with
471 these findings, the cross reactivity of other lateral flow assays specifically designed for AFB1
472 have been reported, which was associated to the structural similarity of mycotoxins,
473 especially aflatoxins (Shim et al., 2014; Zhu et al., 2018; Zhao et al., 2020).

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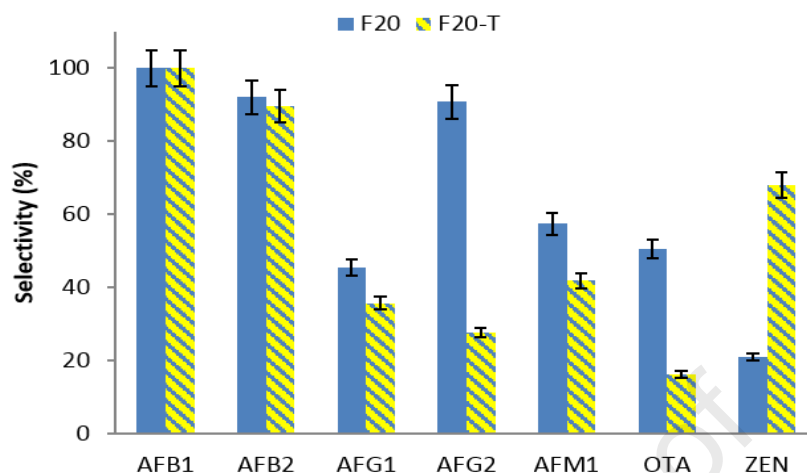
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482

483 Figure 3. Selectivity of F20-T and F20 -based lateral flow strips towards various mycotoxins (10 ng/mL).

484

485 Due to the difficulty in identifying high selectivity binding probes through experimental
 486 methods (Ruscito and DeRosa, 2016), computational simulation techniques can be considered
 487 as promising approaches to find or improve probes toward a specific target (Mousivand et al.,
 488 2020). According to the docking results, F20-T showed the lower binding affinity along with
 489 the smaller binding pocket in complex with other mycotoxins except ZEA (Supplementary
 490 Information, Table S4). It seems that the higher selectivity of F20-T compared to F20 is
 491 associated with the fewer binding sites and possible conformations due to the aptamer
 492 truncation. The *in silico* findings were largely consistent with those experimentally obtained
 493 in terms of selectivity of the aptamers.

494

495 3.9. Real sample analysis

496 The accuracy of both designed aptamer based lateral flow strips were evaluated through
 497 analyzing four HPLC-certified corn flour samples. Although both F20 and F20- T based test
 498 strips were able to detect AFB1 in positive samples under optimum conditions, the parent
 499 sequence based strip was more sensitive in term of recovery percentage (Table 2). According
 500 to the results, the T/C ratios calculated for the sample 1 and the blank sample (0 ng/ml of
 501 AFB1) were relatively similar and estimated as 82.4 and 95.3 % for F20-T and F20 based test
 502 strips respectively. Therefore, the possibility of matrix interference and consequently false
 503 positive response were low in both test strips however the parent based test based strip
 504 showed more accurate results than the truncated based one.

505 Table 2. Recovery percentage of AFB₁ from HPLC certified corn flour samples via F20 and F20-T based lateral
506 flow strips under optimum condition.

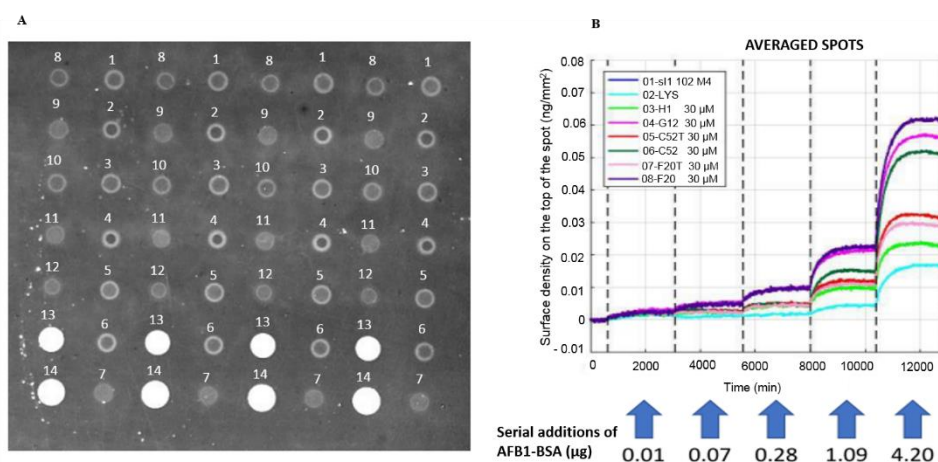
sample	AFB ₁ concentration (ppb) by HPLC	Recovery %	
		F20-T based strip	F20 based strip
2	5	85.0	99.7
3	11.3	77.1	101.0
4	28.9	68.8	110.7

507

508 Furthermore, exploitation of the membrane prepared with lower DNA probe concentration
509 (2.5 μ M) along with longer incubation time (30 min) improved performance of both test
510 strips likely through the improvement of competitive reactions and the reduction of matrix
511 interference, respectively. Regarding to the pre-adsorption of extracted food matrix in the
512 different components of the test strip (Anfossi et al., 2010), it can be interpreted that
513 increasing the membrane incubation time enhanced the sensor performance through matrix
514 effect management. In comparison with the truncated based test strip, all evaluated samples
515 could be correctly ranked based on the AFB₁ concentration values using F20 test strip in
516 various experimentally condition as well (Supplementary Information, Fig. S10).

517 **3.10. AFB₁ binding affinity evaluating through RPI technology**

518 Several surfaces were prepared by immobilizing the provided probes next to control
519 antibodies. After fine-tuning of the microarrays which was necessary for both the deposition
520 process and the surface preparation, microarray surface captured with RPI technology
521 (Giavazzi et al., 2013; Salina et al., 2015). The white spots correspond to a compact single
522 layer of molecules and the signal intensity was proportional to the mass linked to the surface.
523 The black areas correspond to zones without bound molecules. The dissociation constants of
524 various probes were compared through increasing concentrations of ligand (AFB₁-BSA) at
525 fixed times. According to the K_d values and width of plateaus, F20 showed the highest
526 binding affinity over AFB₁ compared to other aptameric probes estimated as 2.11 μ g/mL and
527 83 pg/mm² respectively (Fig 4; Supplementary Information, Figs S11 & S12; Table S9). The
528 lower binding affinity of F20-T compared to the parent sequence confirmed through RPI
529 technology as well. There was a high concordance between the previous studies (Mousivand
530 et al., 2020, 2021) and RPI technology in terms of sorting evaluated aptameric probes based
531 on the K_d values.



532
 533 Figure 4. (A) Microarray surface captured with RPI technology under constant concentration of AFB1-BSA
 534 (12000 µg/ml); probes and their concentrations represent in numbers as follows; 1: (F20; 5 µM), 2: (F20-T; 5
 535 µM), 3: (C52; 5 µM), 4: (C52-T; 5 µM), 5: (g12; 5 µM), 6: (H1; 5 µM), 7: (F20; 30 µM), 8: (F20-T; 30 µM), 9:
 536 (C52; 30 µM), 10: (C52-T; 30 µM), 11: (g12; 30 µM), 12: (H1; 30 µM); 13: (control antibody 1), 14: (control
 537 antibody 2); (B) The graph shows the amount of mass bounded by each type of spot over time at constant
 538 concentration (30 µM) of various aptameric probes; additions are marked with dashed lines.

539

540 Conclusion

541 Given the practical advantages of the aptameric probe technology over antibody generation,
 542 aptamer-based sensors can be considered as promising alternatives for accurate small
 543 molecule monitoring. However, the structural simplicity, few binding sites along with low
 544 molecular weight of small compounds are still the major bottlenecks for aptamer and
 545 aptasensing platform development for these category of compounds (Ruscito and DeRosa,
 546 2016). As complementary options for experimentally small binding aptamer discovery, the
 547 various *in silico* approaches can significantly influenced their research and commercialization
 548 (Mousivand et al., 2020; Ciriaco et al., 2020). In our previous study, a well-known AFB1
 549 aptamer sequence, Apt1, has been exploited to design a high affinity DNA aptamer, F20,
 550 through the *in silico* maturation strategy. Here, we integrated the truncating strategy and
 551 computational simulation studies to develop a new shorter aptamer, F20-T, based on F20
 552 sequence. Both designed AFB1 aptamers were successfully applied as recognition elements
 553 in the lateral flow aptasensors and the RPI platform for simple and rapid AFB1 detecting.
 554 According to the legal requirements of the European Union, the sensitivity reached by both
 555 new lateral flow test strips was suitable for detecting AFB1 via strip reader. Moreover, they
 556 showed better sensitivity compared to an analogous lateral flow strip exploiting the original

557 Apt1 (Zhang et al., 2018 b). Regarding to the high consistency between our experimental and
 558 in silico findings, aptamer engineering through sequence or scaffold refinement can be
 559 considered as a new and promising research field for novel small binding aptamer
 560 development. Low-cost integration of the newly designed probes as recognition elements in
 561 existing aptasensing platforms allow designing various novel aptasensor for small molecule
 562 target monitoring in a green way.

563

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567

568

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710 **Supplementary material**711 **High performance aptasensing platform development through in silico aptamer**
712 **engineering for aflatoxin B1 monitoring**713 **Maryam Mousivand^{*1,2}, Mohammad Javan-Nikkhah², Laura Anfossi^{3*}, Fabio Di**
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738 **Table S1.** Modified aptamers and probe sequences used in this study

aptamers/probes	sequence (5'-3')
thiol-modified aptamer F20	5'-aatgggcacgtgctgcctatatgtgtctcgtgcccttcgctaggcccactaaaaaaaaaaaaaaaaa-SH-3'
thiol-modified aptamer F20-T	5'-aatgggcacgtgctgcctaaaaaaaaaaaaaaaaa-SH-3'
biotin-modified DNA probe1	5'-agtgggcctagcgaaggcagacacacatataggcagcagcgtgccatt-Biotin-3'
biotin-modified DNA probe2	5'-ttttttttttttttt-Biotin3'
aptamer F20	5'-aatgggcacgtgctgcctatatgtgtctcgtgcccttcgctaggcccact-3'
aptamer F20-T	5'-aatgggcacgtgctgcct-3'

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743 **Table S2.** The sequence, minimum free energy of secondary structure formation (ΔG), length (bp), truncated
744 direction and docking score of aptamers in the truncated library.

Aptamers	Sequences (5'-3')	ΔG (Kcal/mol)	Length (bp)	Truncated direction	Docking score
F20	aatgggcacgtgctgcctatatgtgtctcgtgcccttcgctaggcccact	-8.01	50	-	5.68
F20-T	aatgggcacgtgctgccta	-3.51	19	3'	4.17
F20-40-3	aatgggcacgtgctgcctatatgtgtctcgtgcccttcgc	-8.01	40	3'	3.93
F20-30	atgtgtctcgtgcccttcgctaggcccact	-1.97	30	5'	3.72
F20-17	ccttcgctaggcccact	-0.35	17	5'	3.70
F20-40	tgctgcctatatgtgtctcgtgcccttcgctaggcccact	-2.05	40	5'	3.38
F20-16	cttcgctaggcccact	0.36	16	5'	3.06
F20-18	cccttcgctaggcccact	-0.87	18	5'	2.90
F20-18-3	aatgggcacgtgctgcct	-3.51	18	3'	2.87
F20-19	gcccttcgctaggcccact	-0.89	19	5'	2.86
F20-20-3	aatgggcacgtgctgcctat	-3.66	20	3'	2.83
F20-17-3	aatgggcacgtgctgcc	-2.99	17	3'	2.72
F20-10	taggcccact	0.36	10	5'	2.45
F20-30-3	aatgggcacgtgctgcctatatgtgtctcg	-4.14	30	3'	2.39
F20-10-3	aatgggcacg	1.88	10	3'	2.03
F20-20	tgcccttcgctaggcccact	-1.50	20	5'	1.97
F20-15-3	aatgggcacgtgctg	-0.82	15	3'	1.94
F20-16-3	aatgggcacgtgctgc	-1.15	16	3'	1.88
F20-15	ttcgctaggcccact	0.36	15	5'	1.85
F20-30-3-5	tgctgcctatatgtgtctcgtgcccttcgc	-0.05	30	5' & 3'	1.66

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748 **Table S3.** Principal interactions between residues of the truncated aptamers with AFB1.

Aptamers	Carbone and conventional hydrogen binding	Hydrophobic interaction	Electrostatic interaction
	H-Donor-H-Acceptor	Pi-Orbitals- π/σ /alkyl	Negative-Pi-Orbitals
F20-10	G6:H22 - AFB1:O5	G4:C2' - AFB1- π - σ	
	G6:H22 - AFB1:O6	G4 - AFB1/ π -alkyl	
	G6:H1 - AFB1:O6	G4 - AFB1/ π -alkyl	
	G4:C1' - AFB1:O2		
	AFB1:C15 - G4:N3		
F20-15	AFB1:C17 - G4:O3'		
	G10:C5' - AFB1:O4	U11 - AFB1/ π -alkyl	
	U11:C6 - AFB1:O3		
F20-16	AFB1:C15 - G12:OP1		
	AFB1:C17 - C9:O2	A8 - AFB1/ π - π	G6:OP1 - AFB1/ π -anion
F20-17	G5:H21 - AFB1:O6		
	G6:H21 - AFB1:O5		
	AFB1:C15 - G4:O3'	G5 - AFB1/ π - π	
	G5:H21 - AFB1:O6	G5 - AFB1/ π - π	
F20-18	G6:H21 - AFB1:O5		
	AFB1:C15 - G4:O3'		
	AFB1:C17 - G5:O3'		
	A8:H61 - AFB1:O5	C7 - AFB1/ π - π	
F20-19	A8:H61 - AFB1:O6	T14 - AFB1/ π - π	
	AFB1:C15 - G10:O6	T14 - AFB1/ π - π	
	AFB1:C17 - G15:OP2	C7 - AFB1/ π -alkyl	
	G4:H21 - AFB1:O4	A8 - AFB1/ π -alkyl	
	G5:H21 - AFB1:O1	G5:C1' - AFB1/ π -lone pair	
	AFB1:C15 - G6:N3	G5:O4' - AFB1/ π - π	
F20-20		G5 - AFB1/ π - π	
		G5 - AFB1/ π - π	
		G5 - AFB1/ π -alkyl	
		G5 - AFB1/ π -alkyl	
		G6 - AFB1/ π -alkyl	
F20-30	T24:H3 - AFB1:O6	G25 - AFB1/ π - π	
	G25:H21 - AFB1:O5		
F20-40	AFB1:C15 - T24:O3'		
	G4:H21 - AFB1:O1		G5:OP1 - AFB1/ π -anion
F2-10-3	A8:H62 - AFB1:O4	G3 - AFB1/ π - π	
	G3:C1' - AFB1:O2	A2 - AFB1/ π -alkyl	
	T10:C4' - AFB1:O5	G3 - AFB1/ π -alkyl	

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752 **Table S3.** (continued). Principal interactions between residues of the truncated aptamers with AFB1.

F2-20-3	G3:H21 - AFB1	G3 - AFB1/ π -alkyl	
	AFB1:C17 - T6:OP2 AFB1:C17 - T6:O5'	T6 - AFB1/ π -alkyl	C10:OP1 - AFB1/ π -anion
F2-30-3	A22:C1' - AFB1:O6	T17 - AFB1/ π -alkyl	G19:OP1 - AFB1/ π -anion
		A22 - AFB1/ π -alkyl	A22:OP2 - AFB1/ π -anion
		A22 - AFB1/ π -alkyl	
F2-40-3	AFB1:C17 - T27:O4'	C25 - AFB1/ π -alkyl	
	AFB1:C17 - A38:N1	C39 - AFB1/ π -alkyl	
F2-30-3-5	G20:H21 - AFB1:O6	G20 - AFB1/ π - π	
	AFB1:C17 - G20:O3'	G20 - AFB1/ π - π	
		G20 - AFB1/ π -alkyl	
		G20 - AFB1/ π -alkyl	
F2-15-3	G4:H22 - AFB1:O5	T1 - AFB1/ π - π	
	G4:H22 - AFB1:O6		
	G4:H1 - AFB1:O6		
	T2:H3 - AFB1		
F2-16-3	A14:H62 - AFB1:O4	AFB1:C17 - A14/ π - π	
		G9 - AFB1/ π - π	
		G9 - AFB1/ π - π	
		G9 - AFB1/ π - π	
		G9 - AFB1/ π - π	
F2-17-3	G10:H21 - AFB1:O2	G10 - AFB1/ π -alkyl	
	A9:C1' - AFB1:O4	G11 - AFB1/ π -alkyl	
	AFB1:C17 - T4:O3'		
F2-18-3	C1:C1' - AFB1:O4	C3 - AFB1/ π -alkyl	
	AFB1:C15 - C3:O2		
F2-19-3	G8:H21 - AFB1:O5	T5 - AFB1/ π -alkyl	
	G8:H21 - AFB1:O6		
	AFB1:C3 - T10:OP2		
	AFB1:C17 - G8:O5'		
	AFB1:C17 - C9:OP1		

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760 **Table S4.** Binding energy, binding sites, Inhibition constant, type of interactions (number of interactions) and
761 Ref RMSD for the best conformation of F20 and F20-T aptamers docked with AFB₂, AFG₁, AFG₂, AFM₁, OTA
762 and ZEN.

Complex	Binding Energy (kcal/mol)	Binding sites	Inhibition constant (μm)	Type of interactions* (number of interactions)	RMSD (\AA)
F20-AFB2	-9.67	T28, C27,C17,G12	1.58	Hb(12)+E(6)+O(1)	19.2
F20-AFG1	-5.85	C27,T28,C29,G10,G12,C17	18.28	Hg(4)+Hb(8)+O(1)	21.18
F20-AFG2	-7.0	C27,T28,C29,G10,G12,C17	5.24	Hg(3)+Hb(9)+O(1)	21.43
F20-AFM1	-4.3	C27,T28,C29,G10,G12,C17	562.62	Hg(6)+Hb(2)+O(2)	31.75
F20-OTA	-5.72	C27,T28,C29,G10,G12,C17,C16	39.26	Hg(9)+Hb(13)+O(1)	23.15
F20-ZEN	-5.41	G4,G6,T37	90.55	Hg(3)	24.33
F20-T-AFB2	-8.0	G15,C12,C7, A8,T14	1.38	Hg(1)+Hb(4)+E(8)	21.86
F20-T-AFG1	-4.82	G15,C7, A8,T14	290.86	Hb(6)+E(1)	23.28
F20-T-AFG2	-4.73	G15,G10,C7, A8,T14	343.2	Hg(4)+Hb(6)	24.35
F20-T-AFM1	-4.29	G5,G6,G15,C7	712.65	Hg(5)+Hb(1)	24.9
F20-T-OTA	-4.58	A8,G6	440.32	Hg(3)+Hb(2)	28.62
F20-T-ZEN	-5.44	C7,A8,G15	103.12	Hg(3)+Hb(3)	26.98

763 Hg: Hydrogen Bonding Hb: Hydrophobic Bonding E: Electrostatic O: others

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765 **Table S5.** RMSD, RMSF and Rg values (nm) for binding pocket of the aptamer-AFB₁ complexes (C) with

766 respect to the lone aptamers (F) during 50 ns molecular dynamic simulation.

aptamer	RMSD(F)	RMSD (C)	RMSF(F)	RMSF (C)	Rg(F)	Rg(C)
	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)
F20-T	0.42 \pm 0.04	0.31 \pm 0.04	0.24 \pm 0.03	0.15 \pm 0.02	0.78 \pm 0.2	0.81 \pm 0.2
F20	0.59 \pm 0.07	0.39 \pm 0.04	0.24 \pm 0.06	0.15 \pm 0.05	1.03 \pm 0.03	0.84 \pm 0.02

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768 **Table S6.** RMSD, RMSF and Rg values (nm) for all atoms of the aptamer-AFB₁ complexes (C) with respect to

769 the lone aptamers (F) during 50 ns molecular dynamic simulation.

Aptamer	RMSD(F)	RMSD (C)	RMSF(F)	RMSF (C)	Rg(F)	Rg(C)
	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)	(mean \pm SD)
F20-T	0.48 \pm 0.07	0.40 \pm 0.08	0.24 \pm 0.07	0.14 \pm 0.05	1.19 \pm 0.03	1.28 \pm 0.03
F20	0.94 \pm 0.17	1.35 \pm 0.28	0.608 \pm 0.28	0.8 \pm 36	2.48 \pm 0.10	2.34 \pm 0.27

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773 **Table S7.** Comparison of the hydrogen bond interactions (mean \pm SD) and hydrogen bond occupancy of F20-T
774 and F20 aptamers in the complexes with aflatoxin B₁ during 50 ns of MD simulation

Aptamer	Donor	Acceptor	Hydrogen bond occupancy	hydrogen bond interactions (mean \pm SD)
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			(%)	
F20-T	G10 (H21)	AFB1(O3)	4.4	0.28 ± 0.59
	G10 (H21)	AFB1 (O2)	7.2	
	G10 (H1)	AFB1 (O3)	1	
	G10 (H1)	AFB1 (O2)	15.5	
	C7(H61)	AFB1 (O5)	0.1	
F20	T28 (H3)	AFB1 (O1)	1.1	0.04±0.21
	C17 (H41)	AFB1 (O2)	0.1	
	G15 (H21)	AFB1 (O1)	0.1	
	G12 (H21)	AFB1(O6)	1.3	
	G12 (H21)	AFB1(O1)	0.5	
	G10 (H21)	AFB1(O6)	0.8	
	G10 (H21)	AFB1(O5)	0.2	
	G10 (H21)	AFB1(O4)	0.1	
	G10 (H21)	AFB1(O3)	0.2	

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777 **Table S8.** Comparison of the water intermediate interactions (mean± SD) and their occupancy ranges involved
 778 with AFB1 and binding pocket of F20-T and F20 complexes during 50ns of MD simulation

Aptamer	Water molecules-binding pocket		Water molecules-AFB1	
	hydrogen bond interactions(mean± SD)	occupancy range (%)	hydrogen bond interactions (mean± SD)	occupancy range (%)
F20-T	78.42±4.75	1-5.1	2.91±1.15	0.1-1.4
F20	82.78±5.17	1-9.6	3.32±1.25	0.1-0.8

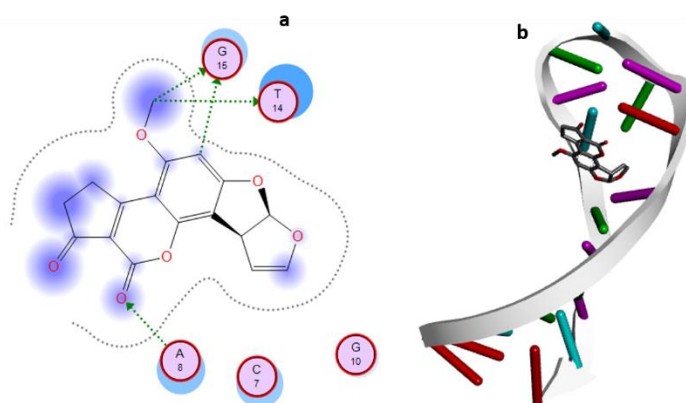
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781 **Table S9.** Determination of dissociation constant (Kd) and width of plateau of F20-T, F20, C52, C52T, g12 and
 782 H1 aptamers via reflective phantom interface (RPI) technology.

Aptamers	Kd (µg/ml)	Kd (nM)	Width of plateau
F20	2.15	31.9	83µg/mm ²
F20-T	4.61	68.8	61µg/mm ²
C52	2.67	39.9	73µg/mm ²
C52T	3.34	52.8	58pg/mm ²
g12	2.12	31.6	78pg/mm ²
H1	3.77	56.1	45pg/mm ²

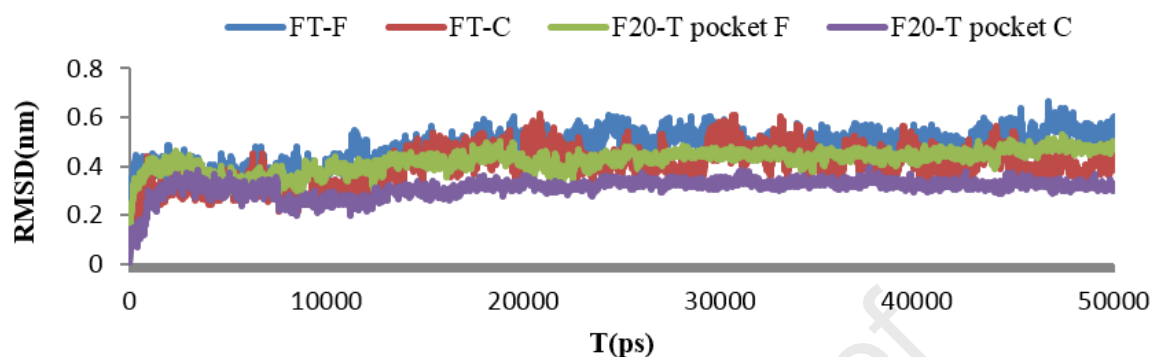
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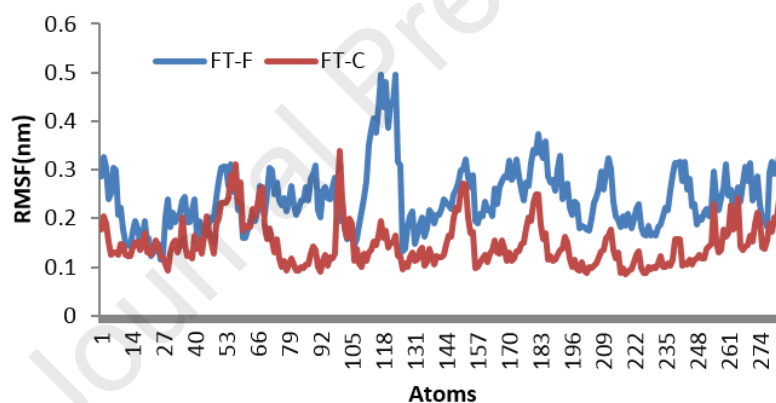
785 **Fig. S1.** The docking results of F20 -T -AFB1 complex and residues involved in binding interaction in 2D (a)
 786 and 3D (b) representation.

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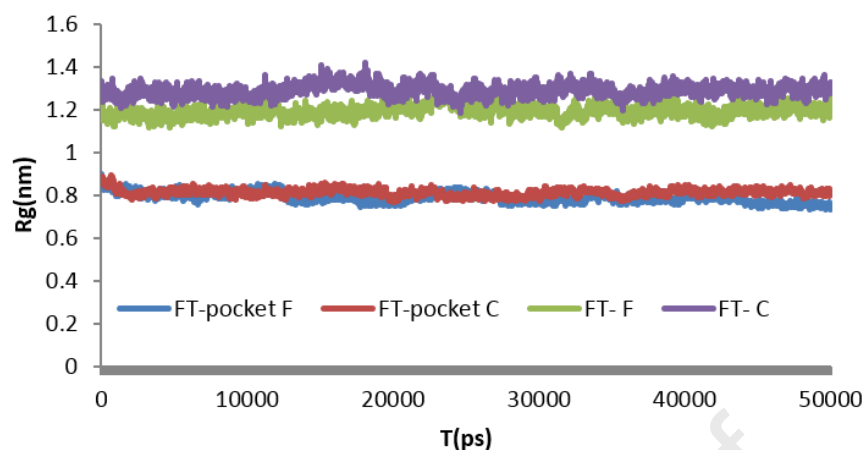
788

789 **Fig. S2.** RMSD plot of F20-T aptamer for all atoms (lone: Blue; in complex with aflatoxin B1: Red) and
 790 binding pocket (lone: Green; in complex with aflatoxin B1: Purple) during the simulation time.



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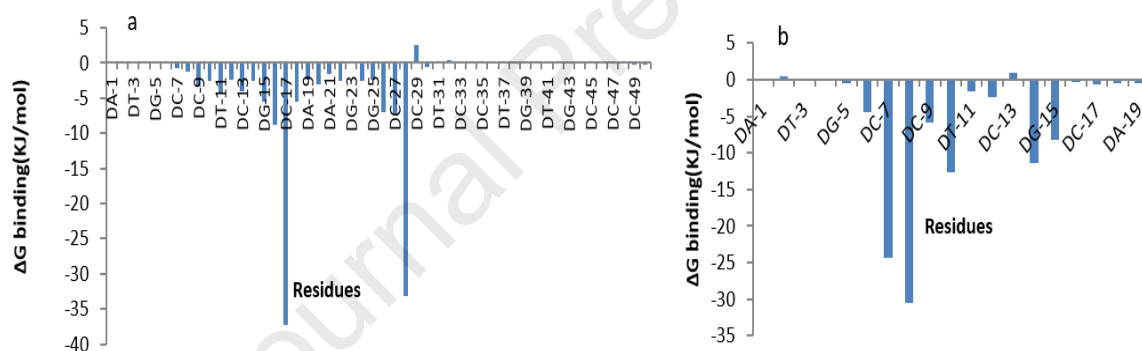
792 **Fig. S3.** RMSF plot of F20-T aptamer for all atoms (lone: Blue; in complex with aflatoxin B1: Red) during the
 793 simulation time.



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795 **Fig. S4.** Rg plot of F20-T aptamer for all atoms (lone: Green; in complex with aflatoxin B1: Purple) and
 796 binding pocket (lone: Blue; in complex with aflatoxin B1: Red) during the simulation time.

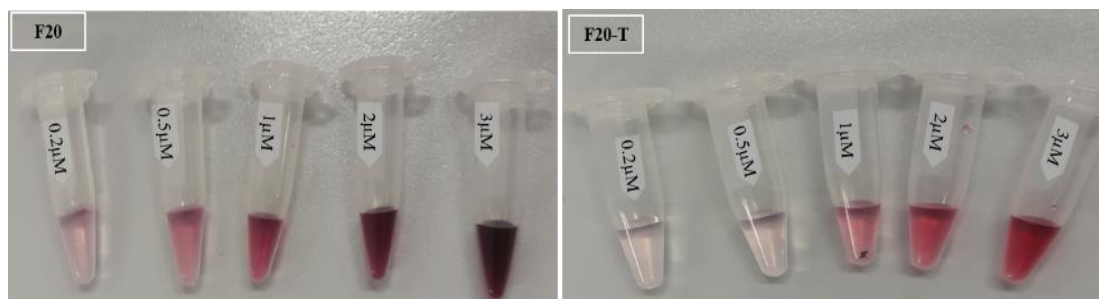
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799 **Fig. S5.** Energy component decomposition analysis per F20 (a) and F20-T (b) residues interacting with aflatoxin
 800 B1 through MM-PBSA method during 50ns of MD simulation.

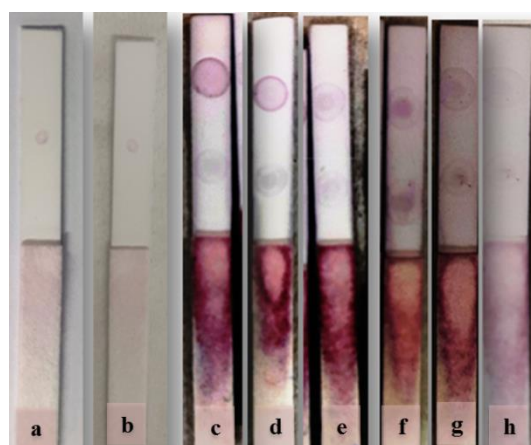
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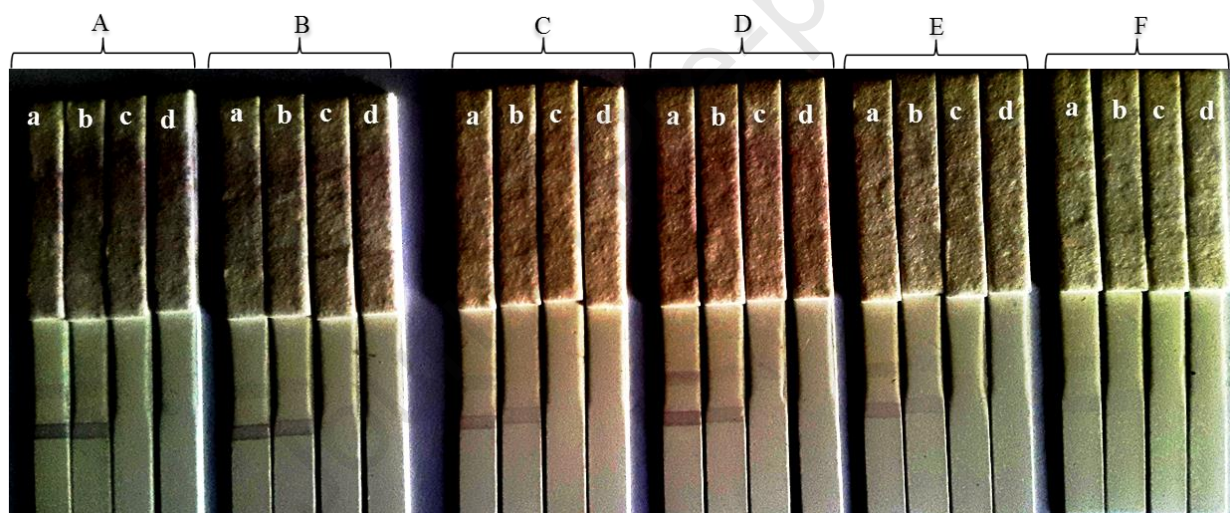
803 **Fig. S6.** Optimization of the gold nanoparticles-aptamer conjugate preparation under different concentrations of
 804 the thiolated F20 and F20-T aptamers.

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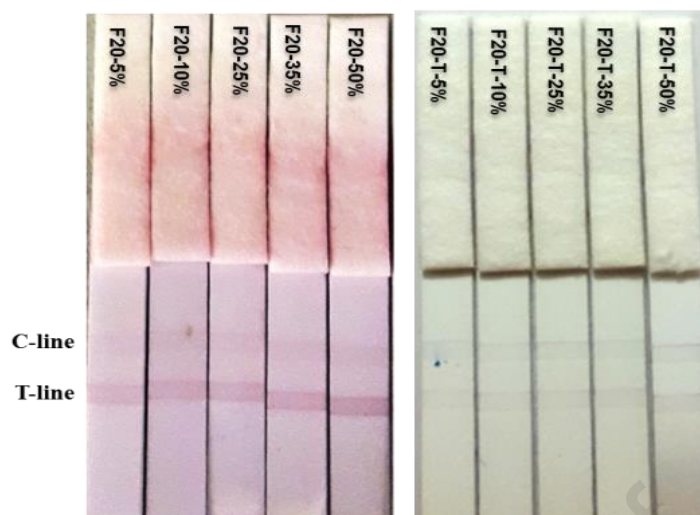
807 **Fig. S7.** Determination of the minimum required concentrations of DNA probe 1 and DNA probe 2 at the test
 808 and control lines under the constant concentration (1 μM) of F20 and F20-T ; a:(F20, 60 μM), b: (F20-T,
 809 60 μM), c:(F20,30 μM), d:(F20,15 μM), e:(F20,5 μM), f:(F20-T, 30 μM), g:(F20-T,15 μM), h: (F20-T, 5 μM)
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812 **Fig. S8.** Optimization of the test and control line performance using various nitrocellulose membranes with
 813 different concentrations of DNA probes 1 and 2 (a:100nM, b:500nM, c:2.5 μM , d:5 μM) under different
 814 concentration of thiolated F20 (A:1 μM ,B:0.2 μM , C:0.1 μM) and F20-T (D: 2 μM , E:0.4 μM , F:0.2 μM)
 815 aptamers.

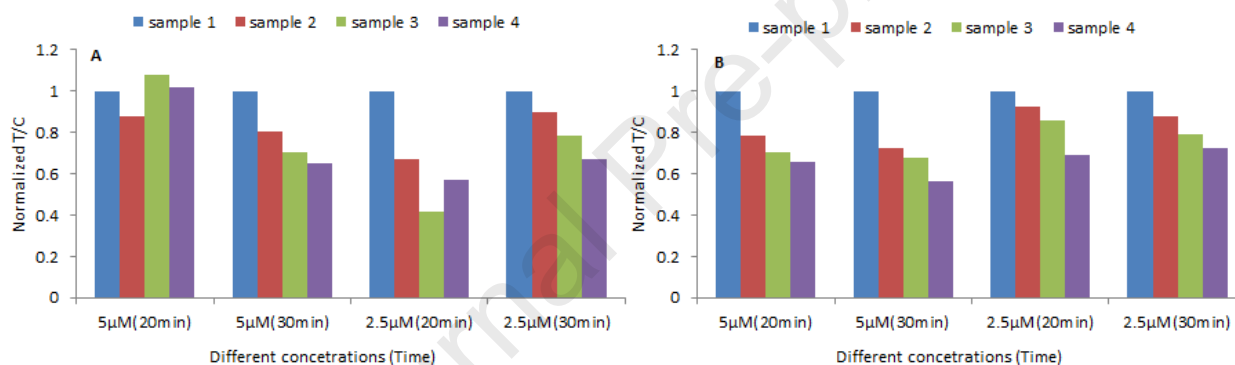
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818 **Fig. S9.** Evaluation of the test and control line intensities of F20 and F20-T based test strips under various
 819 concentrations of aqueous methanol (5, 10, 25, 35, 50% v/v) at AFB₁ constant concentration (10 ng/mL).

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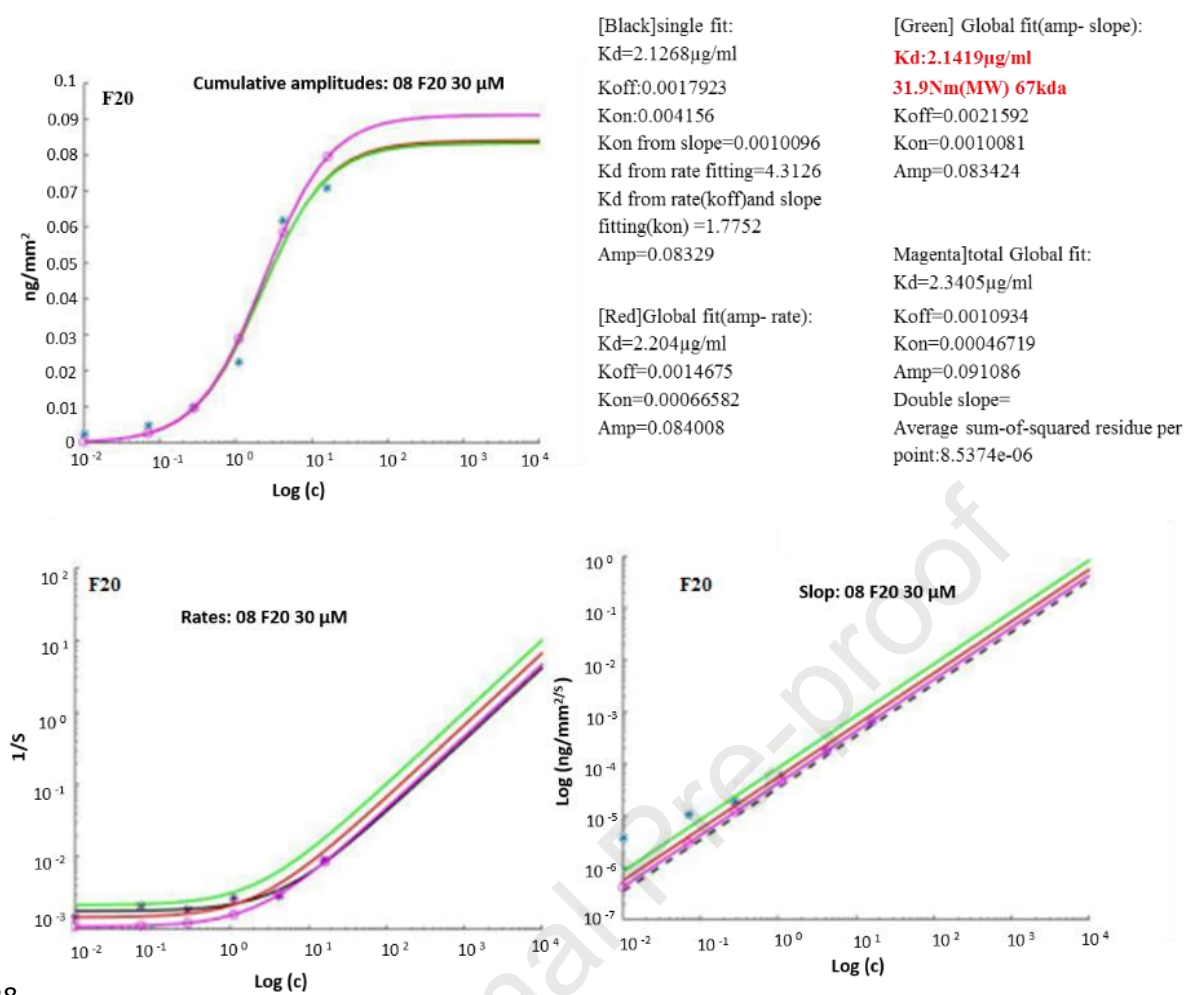


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822 **Fig.S10.** AFB₁ detection in corn flour samples via F20 (A) and F20-T (B) based lateral flow strips under
 823 different conditions including two membranes prepared with different DNA probe concentrations (2.5 and 5
 824 μM) and two incubation time (20 and 30 min). AFB₁ concentrations in HPLC-certified samples 1, 2, 3 and 4
 825 were <LOD, 5, 11.3 and 28.9 ppb, respectively .

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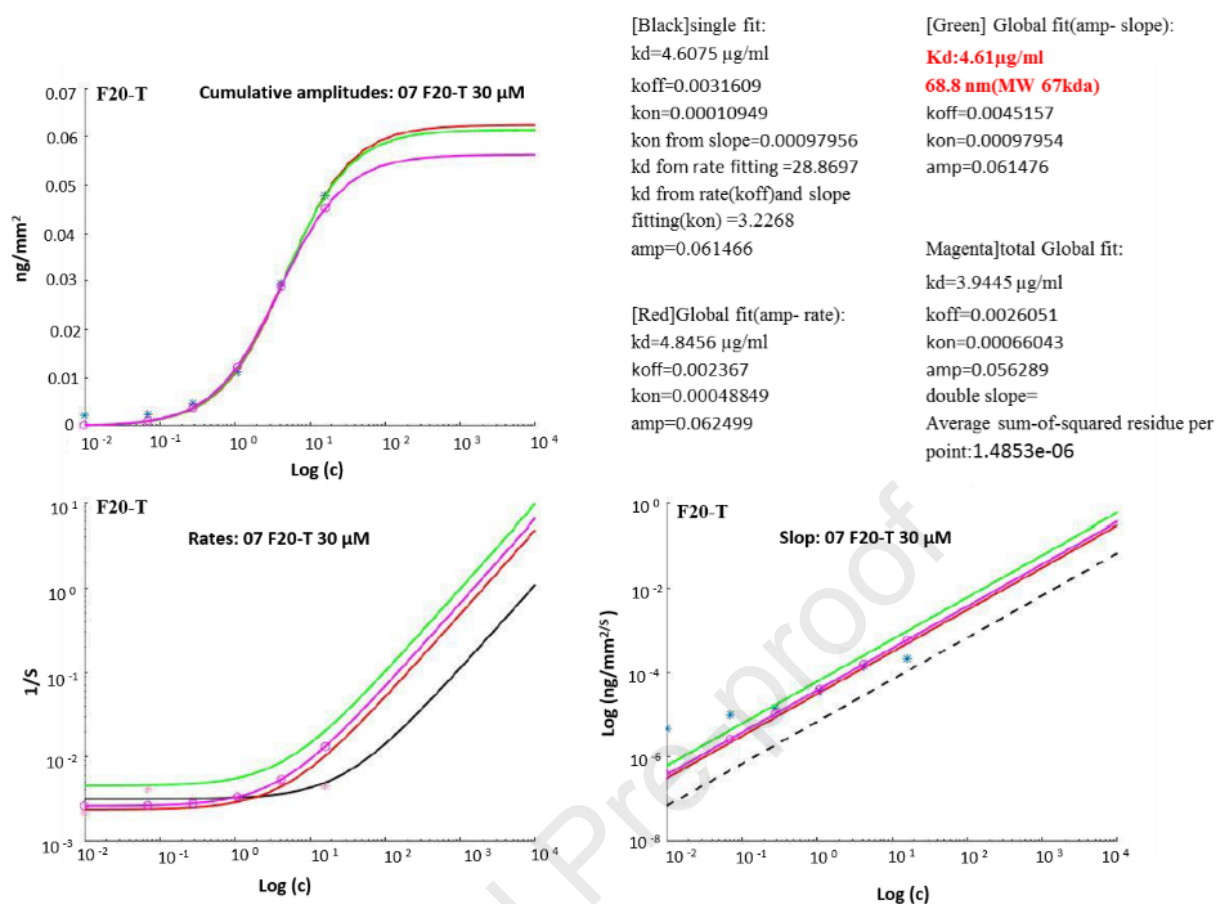
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829 **Fig. S11.** Determination of dissociation constant (K_d) of F20 aptamer via reflective phantom interface (RPI)
 830 technology.

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834 **Fig. S12.** Determination of dissociation constant (K_d) of F20-T aptamer via reflective phantom interface (RPI)
 835 technology.

1. A new truncated Aflatoxin B1 binding aptamer was designed via in silico studies.
2. The truncated and the parent aptamer were successfully applied to build aptasensors.
- 3- The redesigned aptasensing platforms provided high sensitive AFB1 detection.
- 4- In silico engineered aptamers can be costly exploited for new aptasensor development.

Journal Pre-proof

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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