



*Citation for published version:*

Sypabekova, M, Jolly, P, Estrela, P & Kanayeva, D 2019, 'Electrochemical Aptasensor using Optimized Surface Chemistry for the Detection of Mycobacterium tuberculosis Secreted Protein MPT64 in Human Serum', *Biosensors and Bioelectronics*, vol. 123, pp. 141-151. <https://doi.org/10.1016/j.bios.2018.07.053>

*DOI:*

[10.1016/j.bios.2018.07.053](https://doi.org/10.1016/j.bios.2018.07.053)

*Publication date:*

2019

*Document Version*

Peer reviewed version

[Link to publication](#)

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**Electrochemical Aptasensor using Optimized Surface Chemistry for the Detection of *Mycobacterium tuberculosis* Secreted Protein MPT64 in Human Serum**

Marzhan Sypabekova <sup>a, b, d</sup>, Pawan Jolly <sup>c, e</sup>, Pedro Estrela <sup>c\*</sup>, Damira Kanayeva <sup>d\*</sup>

<sup>a</sup> Graduate Program in Science, Engineering, and Technology, School of Engineering, Nazarbayev University, Astana 010000, Kazakhstan

<sup>b</sup> National Laboratory Astana, Nazarbayev University, Astana 010000, Kazakhstan

<sup>c</sup> Centre for Biosensors, Bioelectronics and Biodevices (C3Bio) and Department of Electronic & Electrical Engineering, University of Bath, Calverton Down, Bath BA2 7AY, United Kingdom

<sup>d</sup> Department of Biology, School of Science and Technology, Nazarbayev University, Astana 010000, Kazakhstan

**Present Addresses**

<sup>e</sup> Pawan Jolly has moved to Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, United States

**Corresponding Authors**

\*(P.E.) E-mail: [P.Estrela@bath.ac.uk](mailto:P.Estrela@bath.ac.uk)

\*(D.K.) E-mail: [dkanayeva@nu.edu.kz](mailto:dkanayeva@nu.edu.kz)

25 **ABSTRACT**

26 Tuberculosis (TB) remains one of the leading causes of mortality worldwide. There is a great need  
27 for the development of diagnostic tests, which are reliable, sensitive, stable, and low cost to enable  
28 early diagnosis of TB in communities with scarce resources. This study reports the optimization  
29 and evaluation of a synthetic receptor, an aptamer, for the detection of the secreted protein MPT64,  
30 which is a highly immunogenic polypeptide of *Mycobacterium tuberculosis*, a causative agent of  
31 TB. The study investigates combinatorial effects of an aptamer linker and a co-adsorbent onto a  
32 gold electrode for optimal binding efficiency and reduced non-specific interactions for label-free  
33 detection of MPT64 using electrochemical impedance spectroscopy. Two types of co-adsorbents  
34 and two types of aptamer linkers were studied and high specificity and sensitivity to MPT64 was  
35 observed for a surface prepared with a thiol PEGylated aptamer HS-(CH<sub>2</sub>)<sub>6</sub>-OP(O)<sub>2</sub>O-  
36 (CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>-TTTTT-aptamer and 6-mercaptohexanol in a ratio of 1:100. The developed aptamer-  
37 based sensor was successfully used with spiked human serum sample with a limit of detection of  
38 81 pM. This work demonstrates the use of the MPT64 aptamer as a lower cost, more sustainable  
39 and stable alternative of antibodies for the development of point-of-care TB biosensors decreasing  
40 the detection time from several days or hours to thirty minutes.

41

42 **KEYWORDS**

43 *Mycobacterium tuberculosis*; antigen MPT64; aptamer; surface chemistry; detection; electro-  
44 chemistry

45

46

## 47 **1. Introduction**

48 In 2015 alone, the worldwide tuberculosis (TB) epidemic accounted for 10.4 million new TB  
49 cases, of which 480,000 were multidrug resistant TB, with an estimated 1.4 million deaths (WHO,  
50 2016). TB remains one of the top 10 causes of death worldwide. Despite advances in diagnostics,  
51 a considerable proportion of the TB cases reported are still clinically diagnosed rather than bacte-  
52 riologically confirmed (WHO, 2016). TB detection remains a significant healthcare issue in the  
53 developing world owing to a number of challenges. First of all, *Mycobacterium tuberculosis (Mtb)*,  
54 a causative agent of TB, is a slow-growing bacterium, which takes 4-8 weeks to grow on a tradi-  
55 tional solid medium and 10-14 days even with a rapid liquid culture (Andersen et al., 2000). Sec-  
56 ondly, pulmonary TB presents low clinical symptoms early in the disease course, which leads to a  
57 delay in seeking professional care. Thirdly, active pulmonary TB may present a low bacillary bur-  
58 den at the early stage, which often leads to low sensitivity for sputum smear microscopy, com-  
59 monly used in communities with scarce resources. Therefore, there is a need to develop simple,  
60 easily scalable, and accurate diagnostics based on a novel biomarker discovery and new diagnostic  
61 assays with an emphasis on alternative non-sputum diagnostics targeting blood, urine or breath  
62 (Mehaffy et al., 2017).

63 The MPT64 protein is one of the biomarkers that is significantly highly expressed in individuals  
64 with active TB compared to healthy individuals. The protein is essential for mycobacterial survival  
65 and persistence in the host cell. *Mtb* has the ability to inhibit the apoptosis of infected macrophages  
66 by deactivating the expression of apoptotic cytokines and hence promoting the survival and viru-  
67 lence of the mycobacteria (Kruh-Garcia et al., 2014). Studies showed that exosomes released from  
68 infected macrophages contain mycobacterial proteins, including MPT64. It has been predicted that  
69 the existence of MPT64 and other TB proteins could be associated with their function during the  
70 establishment and maintenance of an intracellular infection. It has also been reported that these  
71 TB-protein enriched exosomes were found to be released into the serum, which opens up the win-  
72 dow for diagnostics based on biomarker identification in serum exosomes to reveal active TB in  
73 patients (Kruh-Garcia et al., 2014). According to the latest multiple reaction monitoring assays,  
74 the clinical diagnostic level of MPT64 protein in isolated exosomes from the serum was at a sub-  
75 nanomolar (nM) range (Mehaffy et al., 2017).

76 Current TB diagnosis based on the detection of MPT64 has been used in skin patch tests, im-  
77 munochromatographic, immunohistochemical, and enzyme linked immunosorbent based assays

78 (ELISA) (Arora et al., 2015; Bekmurzayeva et al., 2013; Kohli et al., 2014; Tadele et al., 2014).  
79 These methods are based on antigen/antibody interactions. Although routinely used, antibodies  
80 have certain limitations in their use in diagnostic tests in low resource settings: expensive produc-  
81 tion, temperature sensitive, easily undergo functional modifications, limited shelf life (less than 6  
82 months), and often there are batch to batch variations. Other detection studies based on reverse  
83 transcription polymerase chain reaction (RT-PCR) targeting the MPT64 gene offer great sensitiv-  
84 ity and specificity (Pinhata et al., 2015; Tadele et al., 2014). However, they are cumbersome in  
85 resource limited settings and require a relatively expensive set of reagents and trained personnel.  
86 Hence, there is a need for the development of an alternative detection methodology for TB.

87 It has been lately demonstrated that aptamers offer great advantages over antibodies in terms of  
88 synthesis scale, cost, and ease of modification. In addition, aptamers have unlimited shelf life,  
89 uniform activity regardless of batch synthesis, and are functional under a broad range of conditions  
90 defined by the user. Hence, they have a potential to find their application in developing aptasensors  
91 for TB detection, where they can serve as bio-recognition elements (Jayasena, 1999; Kuwahara,  
92 2014). The combination of aptamers and sensitive detection methods, such as electrochemical im-  
93 pedance spectroscopy (EIS), would allow increasing the accuracy of the detection. EIS has numer-  
94 ous advantages over other detection techniques. The signal in EIS can be recorded within a small  
95 change of an analyte binding event based not only on molecular interaction level but also on elec-  
96 tron/charge transfer levels. There have only been a few reports on electrochemical detection of  
97 MPT64 antigen using different aptamer sequences (Qin et al., 2009) and various complex surface  
98 chemistries (Bai et al., 2017; Thakur et al., 2017a; Thakur et al., 2017b), including electropoly-  
99 merised poly(3,4-ethylenedioxythiophene) doped carbon nanotubes, gold nanoparticles decorated  
100 with fullerene-doped polyaniline, and graphene modified iron-oxide chitosan hybrid nanocompo-  
101 site film deposited on fluorine tin oxide. Table 1 summarizes currently available TB detection  
102 methods including methods approved by WHO for routine use in anti-tuberculosis dispensaries  
103 and hospitals.

104 In our previous work, an aptamer against MPT64 with a dissociation equilibrium constant  $K_D$   
105 of 8.92 nM was selected using the SELEX (systematic evolution of ligands through exponential  
106 enrichment) technique. The aptamer was also tested for its specificity on clinical sputum samples  
107 (Sypabekova et al., 2017). Here, we report the development of an electrochemical impedance ap-  
108 tasensor for the detection of MPT64 in blood serum. We studied in detail the effect of surface

109 chemistry and aptamer linker molecule type on the sensor performance since the binding perfor-  
110 mance of an aptamer to the target is highly dependent on the type of linker between aptamer and  
111 the surface and also on the co-adsorbent used (Balamurugan et al., 2006). The type of co-adsorbent  
112 influences a considerable reduction of non-specific binding while maintaining the integrity of the  
113 immobilized biomolecules (Nogues et al., 2012). In this work, surface chemistries using co-adsor-  
114 bents 6-mercaptohexanol (MCH) and triethylene glycol mono-11-mercaptopundecyl ether (EG)  
115 were investigated for their antifouling properties with combination of different aptamer linker  
116 types in the form of HS-(CH<sub>2</sub>)<sub>6</sub>-OP(O)<sub>2</sub>O-(CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>-5'-TTTTT-aptamer-3' and HS-(CH<sub>2</sub>)<sub>6</sub>-5'-  
117 TTTTT-aptamer-3' (**Fig. 1**). These linkers were termed as 'long linker' and 'short linker' due to  
118 the addition of the extra -OP(O)<sub>2</sub>O-(CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>- to the former.

119 For the optimized EIS aptasensor in this work, the detection time was significantly reduced  
120 compared to traditional detection times, such as sputum microscopy and PCR, from several hours  
121 and/or days down to 30 min. The surface chemistry used in this study is comparatively simple with  
122 minimal cost, and the detection limit lays well within the current clinical detection range for  
123 MPT64, which is in the nM range (Mehaffy et al., 2017). The optimized surface chemistry pro-  
124 posed in this study can be further exploited in developing MPT64 aptasensors for TB diagnosis  
125 based on biomarker detection.

126

## 127 **2. Material and methods**

### 128 *2.1. Reagents*

129 Thiolated MPT64 aptamers in the form of HS-(CH<sub>2</sub>)<sub>6</sub>-OP(O)<sub>2</sub>O-(CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>-5'-TTTTT-  
130 aptamer-3' (24.3 nmol) were synthesized by Eurogentec (Belgium), and HS-(CH<sub>2</sub>)<sub>6</sub>-5'-TTTTT-  
131 aptamer-3' (47.4 nmol) were synthesized by Sigma Aldrich (UK). Phosphate buffered saline (PBS,  
132 0.01 M, pH 7.4) tablets, human serum albumin (HSA, lyophilized powder, ≥97%), human serum  
133 (from human male AB plasma), trizma base (≥99.0%), hydrochloric acid (HCl, 37%), magnesium  
134 chloride (MgCl<sub>2</sub>, powder, <200 μm), sodium chloride (NaCl, ≥99.5%), sulfuric acid (H<sub>2</sub>SO<sub>4</sub>, 1 M),  
135 ethylenediaminetetraacetic acid (EDTA, 0.5 M in H<sub>2</sub>O, pH 8), triethylene glycol mono-11-mer-  
136 captoundecyl ether (EG), potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) trihy-  
137 drate, hexaammineruthenium (III) chloride (Ru(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup>, 98%) and 6-mercapto-1-hexanol (MCH)  
138 used in the sample preparation were of analytical grade and purchased from Sigma-Aldrich, UK.  
139 Isopropanol (propan-2-ol, HPLC grade), absolute ethanol (HPLC grade) were purchased from

140 Fisher Scientific (UK). Electrode polishing kit was obtained from BASi Inc. (Japan). MPT64 pro-  
141 tein (E5-00061, 1 mg) was purchased from EnoGene (China). Prostate specific antigen (PSA, 100  
142  $\mu\text{g}$ ) and carcinoembryonic antigen (CEA, 1 mg) were purchased from Calbiochem (Canada) and  
143 USBiological (USA), respectively. All aqueous solutions were prepared using 18.2 M $\Omega\text{cm}$  ultra-  
144 pure water with a Pyrogard filter (Millipore, UK).

145

## 146 *2.2. Apparatus*

147 Measurements were recorded using a CompactStat potentiostat (Ivium Technologies, the Neth-  
148 erlands) with Ivium soft Electrochemistry software. A three-electrode cell with a Ag/AgCl refer-  
149 ence electrode (BASi, USA) connected via a salt bridge filled with buffer and platinum counter  
150 electrode (ALS, Japan) were used for all measurements. The electrochemical impedance spectrum  
151 was measured in a buffer containing 2 mM ferro/ferricyanide  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox couple (potas-  
152 sium hexacyanoferrate II/III). The frequency range used for the measurement was in the range of  
153 10 kHz to 100 mHz, with a 10 mV a.c. voltage superimposed on a bias d.c. voltage of 0.2 V vs  
154 Ag/AgCl, corresponding to the formal potential of the redox couple. All measurements were per-  
155 formed at room temperature inside a Faraday cage. All measurements were carried out in triplicate,  
156 and the mean value of replicates, standard deviations and standard errors from the mean were used  
157 to report the results.

158

## 159 *2.3. Electrode surface preparation*

160 Gold disk working electrodes with 2 mm diameter (CH Instruments, TX, USA) were cleaned  
161 mechanically and electrochemically. Electrodes were first cleaned by sonication in absolute etha-  
162 nol for 10 min, then, mechanically polished for 2 min with 1  $\mu\text{m}$  diamond powder and then with  
163 0.05  $\mu\text{m}$  alumina slurry on respective polishing pads. The electrodes were sonicated for 2 min in  
164 isopropanol and for 10 min in Milli-Q water after each step. Electrodes were then cleaned electro-  
165 chemically in 0.5 M  $\text{H}_2\text{SO}_4$  by scanning the potential between oxidation and reduction of gold, -  
166 0.05 and 1.1 V vs. Ag/AgCl for 50 cycles. The electrodes were then thoroughly rinsed with Milli-  
167 Q water and air dried.

168

## 169 *2.4. Co-adsorbent and DNA immobilization*

170 For the development of the MPT64 aptasensor with a co-adsorbent (either MCH or EG), gold  
171 disk working electrodes were incubated for 16 h at 4 °C with a 150 µl mixture of 100 µM of DNA  
172 aptamer (either short linker or long linker) and 100 µM of co-adsorbent, mixed in different ratios.  
173 This step was important for the controlled aptamer immobilization on the surface via thiol bonds.  
174 The combination of the aptamer and a co-adsorbent used in this study is presented in **Fig. 1**. Both  
175 MCH and EG were initially diluted in absolute ethanol at 100 mM to make stock solutions and  
176 stored at -20 °C. Further dilutions of both MCH and EG were prepared prior to incubation in  
177 different measurement buffers: SELEX buffer (50 mM Tris-HCl; 25 mM NaCl; 5 mM MgCl<sub>2</sub>; pH  
178 7.5), 10× diluted SELEX buffer, and PBS buffer (10 mM and/or 1 mM, pH 7.4). All buffer solu-  
179 tions were filtered using a non-pyrogenic sterile polystyrene 500 ml bottle top filter (Corning In-  
180 corporated, USA) and adjusted at room temperature before use. Prior to mixing with co-adsor-  
181 bents, the DNA aptamer was heated for 5 min at 95 °C and slowly cooled down to room tempera-  
182 ture. This step was important to linearize the aptamer sequence and slowly allow it to obtain its  
183 most favorable conformation. After incubation, the electrodes were rinsed with the measurement  
184 buffer to remove any unattached DNA aptamers. A 150 µl of 1 mM of either MCH or EG was  
185 applied onto the surface of the electrodes for another 1 h at room temperature to ensure complete  
186 thiol coverage of the gold surface and to displace non-specific interactions between the DNA and  
187 gold (Jolly et al., 2015; Keighley et al., 2008). After rinsing the electrodes with the measurement  
188 buffer, they were placed into the measurement buffer with 2 mM ferro/ferricyanide [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup>  
189 redox couple for at least 1 h for surface stabilization until a stable EIS signal was obtained. The  
190 stability measurements were performed in the same time interval that were used for incubation  
191 with protein to monitor the drift over time or any changes in EIS due to external factors. Once  
192 stable Nyquist curves were recorded, proteins were then incubated to monitor binding events. The  
193 stability of the signal was an indicator of the fact that the aptamer had a stable conformational  
194 change on the electrode surface with no further fluctuations in EIS signal. The stable signal was  
195 used as a baseline for subsequent protein concentration measurements.

196

### 197 *2.5. Detection of MPT64*

198 Detection of the target MPT64 with the electrochemical aptasensor was achieved by incubating  
199 the MPT64 protein for 30 min at room temperature on the surface of the electrode that was pre-  
200 immobilized with the co-adsorbent and DNA aptamer. For the detection studies, a wide range of



201 MPT64 concentrations were used from 24 pM up to 200 nM. MPT64 protein was diluted in dif-  
202 ferent measurement buffers: SELEX buffer; 10× diluted SELEX buffer; 10 mM PBS; 1 mM PBS;  
203 1/10 and 1/100 diluted human serum samples in SELEX buffer.

204 For the specificity studies different concentrations (0.1, 1, and 10 nM) of the non-target proteins  
205 HSA, CEA, PSA, and target MPT64 protein diluted in SELEX buffer were incubated for 30 min  
206 on the gold electrode surfaces based on a long linker DNA aptamer/MCH surface chemistry at a  
207 ratio of 1/100.

208

#### 209 *2.6. Preparation and measurement of spiked samples with MPT64*

210 Serum samples were filtered using a 0.2 µm sterile syringe (Corning Inc.) to remove large mo-  
211 lecular formations. The serum dilutions (1/100 and 1/10) were made in SELEX buffer. Co-adsor-  
212 bent and DNA immobilized electrodes were first stabilized in diluted serum for at least 1h until a  
213 stable signal was obtained from the EIS measurement. The electrodes were then incubated in di-  
214 luted serum with a wide range of MPT64 protein concentrations (from 24 pM up to 100 nM) for  
215 30 min at room temperature.

216

#### 217 *2.7. BioFET measurements*

218 Biologically-sensitive field-effect transistors (BioFETs) were used to confirm the EIS results.  
219 The BioFET measurements were carried out in an extended gate mode by connecting the gold  
220 electrodes via a metal wire to the gate of an n-type metal oxide semiconductor field-effect transis-  
221 tor (MOSFET) (Formisano et al., 2016). The MOSFET readings were recorded using an Agilent  
222 B1500A Semiconductor Device Analyzer with EasyEXPERT software. The sensor consisted of  
223 thin film gold electrodes (Micrux Technologies, Spain) placed in a flow cell. The FET structure  
224 was used to transduce the binding events on the gold electrode into a shift in the threshold voltage  
225 of the transistor. An Ag wire coated with silver chloride paste (ALS, Japan) served as a reference  
226 electrode. Prior to co-adsorbent/aptamer complex immobilization, electrodes were cleaned by son-  
227 ication in absolute ethanol for 5 min and then in ultrapure water for additional 5 min. The electrode  
228 preparation was the same as described in Section 2.4. Different concentrations (0.001, 0.01, 0.1,  
229 and 1 nM) of MPT64 were diluted in SELEX buffer and measured using the same conditions as  
230 performed for EIS measurements but without the use of redox markers.

231

## 232 2.8. Contact angle measurement

233 An in-house built optical angle setup was used for contact angle measurement after each im-  
234 mobilization and detection steps (Miodek et al., 2015). Due to the inability of the setup to accom-  
235 modate the measurement electrodes, thermally evaporated gold electrodes were used instead. The  
236 electrodes were sonicated in absolute ethanol for 5 min and then in ultrapure water for another 5  
237 min. Electrodes were then exposed to UV/ozone (BioForce Nanosciences ProCleaner, USA) for  
238 30 min. The MCH/aptamer complex was prepared and incubated overnight as described previ-  
239 ously. 50 nM of MPT64 was applied onto the pre-treated surface of the electrode for 30 min, and  
240 50 nM HSA served as a control. Electrodes were then rinsed with ultrapure water and dried under  
241 a gentle air flux. Electrodes were then placed on the stage and a 5  $\mu$ l drop of ultrapure water was  
242 dispensed. The wetting of surface was then captured using a Nikon p520 camera. Contact angle  
243 was measured using an on-screen protractor GNU GPL v3  
244 (<http://osprotractor.sourceforge.net/Protractor.html>).

## 246 2.9. Chronocoulometry

247 The surface density of DNA aptamer immobilized on the electrode was determined using  
248 chronocoulometry (Formisano et al., 2015; Keighley et al., 2008; Steel et al., 1998). The method  
249 was used to determine the optimal ratio for MPT64 detection on the electrode surface at different  
250 ratios of long linker aptamer/MCH (1/50; 1/100; 1/200; 1/500) required for MPT64 binding. The  
251 final thiol concentration was adjusted to 100  $\mu$ M. Electrodes functionalized with DNA aptamer  
252 and MCH were first immersed into 10 mM Tris-HCl (pH 7.4) for 1 h. A potential step from -300  
253 to -800 mV versus Hg/Hg<sub>2</sub>SO<sub>4</sub> was applied for 500 ms, and the resulting charge flow was meas-  
254 ured. The same electrodes were then immersed into 100  $\mu$ M hexaammineruthenium (III) chloride  
255 (Ru(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup>) in 10 mM Tris-HCl (pH 7.4) buffer, and the resulting charge was again measured.  
256 The number of aptamer molecules per electrode area was calculated based on the integrated Cot-  
257 trell equation (Keighley et al., 2008). The DNA surface density was determined from the surface  
258 excess of Ru(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup> as  $\Gamma_{\text{DNA}} = \Gamma_0(z/m)N_A$ , where  $\Gamma_{\text{DNA}}$  is the probe surface density (mole-  
259 cules/cm<sup>2</sup>),  $m$  is the number of negatively charged phosphate groups on the probe DNA,  $z$  is the  
260 charge on the redox molecule, and  $N_A$  is Avogadro's constant. The number of DNA molecules for  
261 each long linker aptamer/MCH ratio was calculated as the mean of three different electrodes based  
262 on the integrated Cottrell equation. For the protein binding studies, the Ru(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup> molecules were

263 removed from the previously used electrodes by incubating and gently shaking the electrodes in  
264 10 mM Tris-HCl with 10 mM EDTA pH 7.4 for 15 min. The electrodes were then rinsed with the  
265 measurement buffer and stabilized for at least 1 h in measurement buffer with 2 mM ferro/ferricy-  
266 anide  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox couple followed by the EIS measurements. The electrodes were then  
267 immersed in different concentrations of MPT64 protein (0.1, 1, 10 nM) for 30 min followed by  
268 rinsing with measurement buffer, which was then ready for a new EIS measurement recording.

269

## 270 *2.10. Statistical analysis*

271 All statistical analyses were performed using Graphpad Prism 6 (Graphpad Software, Inc),  
272 Origin Pro (OriginLab Corporation), and Excel (Microsoft Office Professional Plus 2013).

273

## 274 **3. Results and discussion**

### 275 *3.1. Surface chemistry optimization studies*

276 The type and the length of the aptamer linker and co-adsorbent were shown to be important for  
277 the aptasensor development (Balamurugan et al., 2006). **Fig. 1** presents a schematic overview of  
278 the sensor surface chemistries applied in this study, which includes three different types based on  
279 either DNA aptamer with short or long linker and MCH and EG as a co-adsorbent. Based on pub-  
280 lished work on antifouling properties of aptamers with the linker and the EG-type co-adsorbent  
281 (Nogues et al., 2012), the combination of short linker DNA and EG as a co-adsorbent was not  
282 tested in the current study. This combination is likely to hinder or diminish the availability of the  
283 short linker aptamer to MPT64 and make the aptamer inaccessible and/or unavailable to the  
284 MPT64 target.

285 We previously reported a study on selection, characterization and application of DNA aptamers  
286 for detection of *Mtb* secreted protein MPT64, where aptamers were selected using the SELEX  
287 method and tested on clinical sputum samples using enzyme linked oligonucleotide assay  
288 (ELONA) with sensitivity and specificity of 91.3% and 90%, respectively (Sypabekova et al.,  
289 2017). The total length of the DNA aptamer was 40 nucleotides, which was modified in this study  
290 by the addition of the long linker in the form of HS-(CH<sub>2</sub>)<sub>6</sub>-OP(O)<sub>2</sub>O-(CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>-TTTTT-  
291 aptamer and the shorter linker HS-(CH<sub>2</sub>)<sub>6</sub>-TTTTT-aptamer. The linkers and five thymine residues  
292 were included to provide spacing between the aptamer and the surface so that it can easily fold and

293 capture the protein. The long linker was made of additional ethylene oxide (CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>, a hydro-  
294 philic part of the linker. This group is essential for protruding the aptamer from the monolayer  
295 surface and, thereby, avoiding any steric hindrance associated with protein binding. The ethylene  
296 oxide also reduces non-specific binding on the electrode surface (Jolly et al., 2016a; Jolly et al.,  
297 2016c). Both MCH and EG are simple and common chemicals that have been used as co-adsor-  
298 bents in surface functionalization and in the detection of molecules.

299 To determine which surface chemistry would provide better binding to our protein of interest,  
300 the BioFET was recorded after incubating the electrode surface with target MPT64. The BioFET  
301 allows the immobilization of an aptamer layer onto an extended gate of a MOSFET and enables  
302 fast quantification of an electrical charge variation arising from protein binding. With the BioFET  
303 technique, very low limits of detection can be obtained and hence can be used for confirmation of  
304 the results obtained from EIS. **Fig. 2a** illustrates a typical transfer characteristic (drain current,  $I_D$ ,  
305 vs. gate-to-source voltage,  $V_{GS}$ ) curve for an electrode before and after MPT64 binding event onto  
306 the surface of the functionalized gold electrode. Depending on the surface charges of the protein,  
307 the BioFET can produce a positive or negative shift in its transfer characteristic. Any changes in  
308  $V_{GS}$  modulate the threshold voltage of the transistor and thereby alter the drain current  
309 (Aliakbarinodehi et al., 2017). Surface charge analysis for MPT64 previously showed that posi-  
310 tively charged hydrophilic amino acids, such as arginine and lysine, were found to be grouped on  
311 the surface of the protein. Hence, the positive charge of MPT64 produced a negative shift in the  
312  $V_{GS}$  of the BioFET as can be seen in **Fig. 2a**. **Fig. 2b** presents the values of  $\Delta V_{GS}$  for different  
313 MPT64 concentrations (0.001, 0.01, 0.1, 1 nM) on different surface modifications onto the IDE  
314 gold electrode: i) a short linker aptamer/MCH, ii) a long linker aptamer/EG, and iii) a long linker  
315 aptamer/MCH. The  $\Delta V_{GS}$  for all three surface chemistries was determined at  $I_D=22.5 \mu\text{A}$ . The  $\Delta V_{GS}$   
316 for the long linker aptamer/MCH complex increased from -17.35 mV to -42.13 mV when the  
317 MPT64 concentration increased from 1 pM to 1 nM. The combination of the two other surface  
318 chemistries (long linker DNA aptamer/EG and short linker DNA aptamer/MCH) studies showed  
319 inconsistent changes in  $\Delta V_{GS}$  (**Fig. 2b**) with large error bars. This inconsistency could be due to  
320 non-specific binding of the target protein to the functionalized surface and reduced reproducibility.  
321 The BioFET data suggests that the surface chemistry based on the long linker DNA aptamer/MCH  
322 (**Fig. 1c**) would provide better antifouling properties for MPT64 binding, better reproducibility, as  
323 well as improved specificity towards the protein detection.

324 We also recorded the EIS signal for the three surface chemistries: short linker DNA ap-  
325 tamer/MCH, long linker DNA aptamer/MCH, and long linker DNA aptamer/EG. The surface  
326 chemistry based on a short linker-DNA aptamer/MCH (**Fig. 1a**) showed a concentration dependent  
327 increase in charge transfer resistance  $R_{ct}$  change (3.9%, 7.5%, and 13.3% at 0.1, 1, and 10 nM of  
328 MPT64, respectively) (see Supplementary Information, **Fig. S1**). The same surface chemistry  
329 showed a non-specific interaction with HSA (with a  $R_{ct}$  change between 2.3% at 0.1 nM and 12.3%  
330 at 10 nM). The results for this specific surface chemistry showed that the short linker ap-  
331 tamer/MCH approach, although sensitive to MPT64, it does not prevent the binding of interferent  
332 molecules, hence making it an unreliable surface for use with complex samples. In addition, the  
333 large error bars in **Fig. S1** (Supplementary Information) are indicative of electrode to electrode  
334 variability. The surface chemistry based on the long linker DNA aptamer/MCH (**Fig. 1c**), meas-  
335 ured with redox markers in solution, showed a  $R_{ct}$  signal change that increases with MPT64 con-  
336 centration (5.8%, 12.0%, and 21.2% for 0.1, 1, and 10 nM of MPT64, respectively), whereas it  
337 remained almost unchanged (less than 4%) when incubated with HSA up to a concentration of 10  
338 nM (Supplementary Information, **Fig. S1**). These measurements were recorded upon a 1/100 ratio  
339 of long linker DNA aptamer/MCH. This significant increase in  $R_{ct}$  signal from 5.8% up to 21.2%  
340 for MPT64 compared to other surface chemistries studies could be due to the blocking effect to  
341 electron transfer after attachment of protein onto the surface of the modified electrode. Another  
342 reason could be due to the extended nature of the aptamer that forms a brush like conformation,  
343 which protrudes from the short MCH monolayer (Nogues et al., 2012). Thereby, the freely moving  
344 extended nature of the aptamer with the long linker enabled MPT64 to bind more efficiently com-  
345 pared to other surface chemistry studies and reduced a non-specific binding. The long linker-DNA  
346 aptamer/EG based surface chemistry (**Fig. 1b**) promotes the formation of an insulating surface,  
347 not suitable for Faradaic measurements. Therefore, measurements were conducted without the use  
348 of the redox probes (i.e. in a non-Faradaic mode) to evaluate the capacitive processes on the sur-  
349 face. A complex capacitance was calculated and used in order to evaluate the capacitance from the  
350 impedance data (Keighley et al., 2008). The signal at the beginning of the recording was quite  
351 unstable and no significant changes were observed in the capacitance upon MPT64 binding (Sup-  
352 plementary Information, **Fig. S2**). Aptamers with longer linker type previously exhibited a 4-fold  
353 increase in binding capacity compared to those with shorter linkers (Balamurugan et al., 2006).  
354 Therefore, the surface chemistry based on the long linker DNA aptamer/EG did not show any

355 sensitivity towards MPT64 nor specificity (data not shown); hence, it was concluded not to use  
356 this surface chemistry for further MPT64 detection. As the result of both BioFET and EIS meas-  
357 urements, the surface chemistry based on the long linker DNA aptamer/MCH was chosen for fur-  
358 ther analysis.

359

### 360 *3.2. Contact angle measurement*

361 The contact angle measurement was used to validate each functionalization step in the aptasen-  
362 sor development and to demonstrate the binding between the target and modified surface. The  
363 contact angle was determined at different stages of the aptasensor development. **Fig. S5** presents  
364 the obtained results (see Supplementary Information; the contact angle images are shown in **Fig.**  
365 **S6**). The surface of the electrode was hydrophilic for all measurements because of UV/ozone ex-  
366 posure during electrode surface preparation and cleaning steps. The contact angle of the bare gold  
367 electrode was  $16.3^\circ$  and an increase to  $21.2^\circ$  was observed after the MCH functionalization step.  
368 The contact angle value for the aptamer/MCH at 1/100 ratio was lower ( $20.5^\circ$ ) compared to MCH  
369 alone indicating on a successful immobilization of aptamers on the surface. The subsequent inter-  
370 action with 50 nM MPT64 target significantly lowered the value of the contact angle down to  
371  $16.5^\circ$ , demonstrating a specific binding to immobilized aptamers compared to control protein, HSA  
372 ( $19.7^\circ$ ). A small non-specific binding of HSA to the MCH treated surface was also shown in pre-  
373 vious studies (Balamurugan et al., 2006; Jolly et al., 2015; Jolly et al., 2016b). However, the change  
374 for non-specific binding was much lower than for specific binding and could be attributed to noise.

375

### 376 *3.3. Long linker DNA aptamer/MCH ratio optimization*

377 The aptamer surface density on the gold electrode and aptamer geometry/positioning is ex-  
378 tremely important for consequent target binding. Therefore, finding the best ratio of an aptamer  
379 and a co-adsorbent is essential for the aptasensor functionalization and development. Since the  
380 long linker aptamer/MCH complex based surface chemistry showed the highest signals in BioFET  
381 and EIS measurements, it was used further in the optimization analysis. A chronocoulometric  
382 method was used to determine the surface density of DNA aptamer (Keighley et al., 2008) so that  
383 the optimal aptamer/MCH ratio is obtained for maximum signal upon MPT64 binding. The method  
384 was based on the electrostatic interaction or intercalation of specific redox cations ( $\text{Ru}(\text{NH}_3)_6^{3+}$ )  
385 with the DNA's sugar-phosphate backbone. The amount of trapped redox marker at the DNA-

386 modified electrode then determined using chronocoulometry. The surface density of the probe was  
387 calculated assuming complete charge compensation of the DNA phosphate residues by redox cat-  
388 ions (Steel et al., 1998). The long linker DNA aptamer was co-immobilized with MCH onto the  
389 gold electrode, where the probe surface density was controlled by varying ratios of DNA aptamer  
390 and MCH in solution. A typical charge flow graph (Supplementary Information, **Fig. S3a**) and  
391 chronocoulometric response curve (Supplementary Information, **Fig. S3b**) with and without  
392  $\text{Ru}(\text{NH}_3)_6^{3+}$  for the long linker DNA aptamer/MCH at 1/100 ratio showed the difference in charge  
393 flow. The data from the graphs was used to determine the DNA aptamer density using the inte-  
394 grated Cottrell's equation (Keighley et al., 2008).

395 The variation of the surface density of the long linker DNA aptamer at different ratios of MCH  
396 is shown in **Fig. 3a**, where four DNA aptamer/MCH ratios were tested: 1/50, 1/100, 1/200, and  
397 1/500. The DNA aptamer density increased as the fraction of DNA to MCH increased from  
398  $6.50 \times 10^{11}$  molecules/cm<sup>2</sup> at 0.002% (1/50 aptamer/MCH) fraction of DNA up to  $7.91 \times 10^{11}$  mole-  
399 cules/cm<sup>2</sup> at 0.02% (1/500 aptamer/MCH) DNA (Table 2). The aptasensor was further tested on  
400 target binding at three different MPT64 concentrations (0.1, 1, and 10 nM) by impedance meas-  
401 urements and the results were compared for different aptamer/MCH ratios (**Fig. 3b**). By analyzing  
402 the  $R_{ct}$  change responses from the EIS signal, the four aptamer/MCH ratios showed different levels  
403 of  $R_{ct}$  change after incubating with MPT64. A significant increase in MPT64 binding was recorded  
404 at an aptamer/MCH ratio of 1/100 by the EIS compared to other ratios studied; the  $\Delta R_{ct}$  signal  
405 increased in the ratio sequence  $1/500 < 1/50 < 1/200 < 1/100$ . A similar trend was also reported  
406 for aptasensors against prostate specific antigen (Formisano et al., 2015). The 1/100 ratio with  
407 aptamer  $7.66 \times 10^{11}$  molecules/cm<sup>2</sup> showed the highest  $R_{ct}$  shift: 3.0%, 10.4%, and 24.7% upon  
408 MPT64 binding at concentrations 0.1, 1, and 10 nM, respectively. However, the 1/50 ratio showed  
409 lower  $R_{ct}$  change (0.01%, 1.6% and 11.6% at 0.1, 1 and 10 nM, respectively) compared to the 1/100  
410 ratio, and it didn't follow the same trend as with the other ratios studied. This might indicate that  
411 the surface was too densely packed, which lowered the sensitivity towards the MPT64 at 1/50 ratio  
412 due to steric hindrance effects. Taking into consideration the long linker that was attached to the  
413 aptamer and the size of the aptamer itself, which was comparatively long (40 nucleotides), the  
414 densely packed surface disabled the aptamer from specific binding to the MPT64. This could be  
415 due to the fact that aptamer configuration did not have a freely moving brush-like confirmation or  
416 it was due to the charge effects from the DNA at 1/50 ratio. Since it is extremely important to find

417 the balance which gives an optimal signal, a ratio of 1/100 was chosen for further sensor develop-  
418 ment.

419

#### 420 3.4. Concentration dependent analysis and specificity studies

421 A schematic overview that represents a binding event of the target MPT64 over the pretreated  
422 gold surface of the electrode with the long linker DNA aptamer/MCH is depicted in **Fig. 4a**. The  
423 typical Nyquist plot in the measurement buffer for an electrode with a bare surface, immobilized  
424 with the long linker DNA aptamer/MCH before and after interaction with 50 nM MPT64 for 30  
425 min is presented in **Fig. 4b**. The EIS signal for the aptamer modified electrode was recorded re-  
426 peitively in 30 min intervals until a stable signal was obtained. This stable signal as used as a  
427 baseline and all the protein incubation signals are reported with respect to this baseline. Any signal  
428 arising from a possible conformational change of the aptamer in the presence of the measurement  
429 solution itself is therefore already included in the baseline.

430 A concentration dependent curve for different MPT64 concentrations using a long linker DNA  
431 aptamer/MCH in a ratio of 1/100 relative to blank measurements from the EIS signal measured in  
432 SELEX buffer is presented in **Fig. 4c**. A concentration dependent analysis was performed for  
433 MPT64 concentrations ranging from 49 pM to 200 nM and showed an increase in charge transfer  
434 resistance ( $R_{ct}$ ) change upon increasing the target concentration. A plateau was reached at 50 nM  
435 concentration of the target MPT64 indicating that the surface was saturated above this concentra-  
436 tion. A linear correlation was found between  $R_{ct}$  change value and target protein in the range of 1-  
437 50 nM. The regression equation for  $R_{ct}$  change versus MPT64 concentration was  
438  $y=0.3395x+11.735$  with  $R^2=0.97$ , where  $x$  is the concentration of MPT64 in nM (**Fig. 4c** inset).  
439 **Fig. 4d** presents an experimental and fitted Nyquist plot for binding of MPT64 at concentrations  
440 ranging from 49 pM to 50 nM using the long linker DNA aptamer/MCH in a ratio of 1/100. A  
441 Randles fitting circuit was used in this study to quantify the target analyte (Liang et al., 2013). **Fig.**  
442 **4d** inset shows the Randles equivalent circuit model selected to fit the experimental data, in which  
443  $R_s$  is the solution resistance connected in series with a constant phase element  $CPE$  and in parallel  
444 with the charge transfer resistance  $R_{ct}$  of the surface and Warburg element  $W$  to model diffusion.

445 The aptasensor based on the long linker DNA aptamer/MCH surface chemistry (**Fig. 1c**), meas-  
446 ured in solution with redox markers, showed a  $R_{ct}$  signal change that increased by 5.82% at 0.1  
447 nM, 12% at 1 nM, and 21.2% when it was incubated with 10 nM MPT64, whereas it remained less



448 than 4% at the same concentrations (0.1, 1, and 10 nM) for the control proteins HSA, CEA, and  
449 PSA showing specificity towards MPT64 (**Fig. 5**). A non-target protein such as CEA (180 kDa)  
450 was selected in this study because it is a member of a family of cell surface glycoproteins that are  
451 produced in excess in human colon carcinomas (Benchimol et al., 1989) with normal level in  
452 healthy individuals being at <5 ng/ml (Asad-Ur-Rahman and Saif, 2016), which is equivalent to  
453 0.02 nM. PSA (30 kDa) is serine protease produced at high concentrations by normal and malig-  
454 nant prostatic epithelium (Stenman et al., 1999) with normal levels in healthy individuals being at  
455 4 ng/ml (Thompson et al., 2004), which is equivalent to 0.13 nM. The maximum concentration  
456 used for the specificity study in this work was 10 nM, which is 500 times higher for CEA and 77  
457 times higher for PSA than the normal clinical range. Therefore, the use of higher concentrations  
458 for CEA and PSA could account for combinatorial interference effects of these proteins on MPT64  
459 detection as opposed to normal level standards. HSA is the most abundant protein in plasma com-  
460 promising about half of serum proteins with a molecular weight of 66.5 kDa (He and Carter, 1992)  
461 with average concentration being at 35 mg/ml (Doumas et al., 1971). All control proteins were  
462 used as a control at the same concentration to confirm that selected aptamers are indeed specific  
463 to MPT64. Upon exposing the aptasensor to 10 nM HSA in measurement buffer, an increase in  
464 the  $R_{ct}$  value of around 4-5% was recorded (**Fig S1, Fig 5.**). Such an effect could be a blocking  
465 effect due to occupancy of free MCH spaces on the sensor surface. The experiments with human  
466 serum samples presented in Section 3.6 show that the change upon MPT64 binding is consistent  
467 with that found in measurement buffer despite of serum being rich on other proteins, including  
468 HSA.

469 In our previous study on selection, characterization, and application of DNA aptamers for de-  
470 tection of *Mtb* secreted protein MPT64, bioinformatics surface charge analysis (PDB code 2hhi)  
471 showed that positively charged hydrophilic amino acids, such as arginine (ARG85 and ARG117)  
472 and lysine (LYS118) were grouped on the target MPT64 model, allowing a potential binding site  
473 for the negatively charged DNA backbone with common TCCAGT located within the biggest  
474 potential stem loop (hairpin), formed by four Watson-Crick base pairs in stem parts and having six  
475 bases in loop part (Sypabekova et al., 2017). A computed GC/AT ratio  $(G + C)/(A + T)$  of the  
476 selected MPT64 aptamer had about 1.11 that provided basic sequence characteristics in terms of  
477 nucleotide composition. This work could provide an understanding of how the target MPT64 and

478 aptamer bind and a study, such as analysis of crystal structure of the protein/aptamer complex  
479 needs to be done to understand the exact mechanism of binding.

480

### 481 3.5. Buffer optimization studies

482 Electrodes functionalized with the long linker-aptamer/MCH at optimized ratio of 1/100 were  
483 further used for buffer optimization studies using the EIS technique. These buffers include the  
484 measurement buffer (SELEX buffer; pH 7.5) in which the aptamer was initially synthesized  
485 (Sypabekova et al., 2017), 10 times diluted SELEX buffer (pH 7.5), 10 mM PBS (pH 7.4), and 1  
486 mM PBS (pH 7.4). The reason for diluting buffers was to reduce the amount of charge screening  
487 during the detection and examine whether dilution influenced the binding efficiency. Upon dilu-  
488 tion, the pH of buffers was adjusted back to the original value. Preparation of 1/100 aptamer/MCH,  
489 stabilization of the electrode surface in the measurement buffer and EIS measurements and rinsing  
490 in between steps were carried out as described in Section 2.4 and were all performed in each type  
491 of buffer. These buffers were used in rinsing steps, in recording the EIS signal, as well as in diluting  
492 the target MPT64. A concentration dependent curve of  $R_{ct}$  change with different concentrations of  
493 MPT64 measured in SELEX buffer is presented in Supplementary Information, **Fig. S4a**. The  
494 increase in  $R_{ct}$  signal and relatively small error bars for the SELEX buffer was consistent with the  
495 increase of protein concentration. When the SELEX buffer was diluted 10 times, the recorded  
496 signal change was below zero at lower MPT64 concentrations (from 100 pM to 1 nM) (Supple-  
497 mentary Information, **Fig. S4b**). The reason for such a drop in the recorded signal value could be  
498 the result of combination of different processes including the change in aptamer conformation  
499 upon binding to MPT64. The magnesium ions within the SELEX buffer are essential for DNA to  
500 form a stable structure (Serec et al., 2016); therefore, the decrease in magnesium might have hin-  
501 dered the proper aptamer folding and eventually resulted in screening of negative charges of DNA  
502 aptamers resulting in reduced  $R_{ct}$  change. An increase in  $R_{ct}$  was observed with higher MPT64  
503 concentrations (<1 nM) due to the binding event of the protein onto the electrode surface, which  
504 disabled the access of the redox probe from the close proximity of the electrode surface. In addi-  
505 tion, batch-to-batch variations were observed, which led to having a high standard deviation at  
506 each sample point; this could be a result of differences in the initial  $R_{ct}$  values from each electrode  
507 at the same protein concentrations. These variations are due to the destabilization of the ap-

508 tamer/protein complex formation. Consequently, there were significant differential values in over-  
509 all  $R_{ct}$  change. Next, measurements were performed in PBS buffer, a widely used buffer in the  
510 development of aptasensors. A seemingly random change in  $R_{ct}$  at 10 mM PBS (Supplementary  
511 Information, **Fig. S4c**) could be explained by the unstable interaction of MPT64 molecules with  
512 the electrode surface. We presume that there was the promotion of non-specific and unstable pro-  
513 tein-protein interactions. The same scenario was observed with 1 mM PBS; although with de-  
514 creased  $R_{ct}$  change (<15%) (Supplementary Information, **Fig. S4d**). Clearly, results of this study  
515 indicated the importance of the buffer in the development of sensitive and specific aptasensor for  
516 the detection of *Mtb* secreted protein MPT64 and showed that the buffer, where aptamers have  
517 been previously raised, could give a better and consistent signal response.

518

### 519 3.6. Analytical evaluation of the aptasensor using serum samples

520 The developed aptasensor with optimized surface chemistry was tested on human serum sam-  
521 ples using EIS. For the determination of the MPT64 in serum, the established standard curve of  
522 the aptamer sensor in buffer solution was not used; rather the standard curve of the aptamer sensor  
523 in serum was used. The standard curve for the aptamer sensor in serum was established by stabi-  
524 lizing the electrode surface in serum to have a good calibration signal. The electrode surface was  
525 primarily stabilized with 1/100 diluted serum until obtaining a stable signal. There are other mol-  
526 ecules such as albumins within the serum that could be absorbed on MCH, hence, the stabilization  
527 step is important to have a good calibration signal. The electrochemical impedance signal in-  
528 creased when the aptamer-modified electrode was immersed in serum when compared to the re-  
529 sults obtained with the buffer (**Fig.6a**). In serum, which contains high concentrations of HSA as  
530 in the normal clinical range, the optimized surface chemistry showed a non-specific binding with  
531  $R_{ct}$  change of around 5-6% without going beyond during repetitive measurement in serum alone  
532 (**Fig.6b**, dashed line). This suggests a possible resilience to the non-specific binding of serum pro-  
533 teins.

534 After a signal stabilization, the electrode was incubated in human serum with a wide range of  
535 MPT64 concentrations (from 12 pM to 100 nM) (**Fig. 6b**). There was an increase in  $R_{ct}$  with an  
536 addition of the target MPT64 at different concentrations in 1/100 diluted serum samples (**Fig.6**).  
537 The sensor demonstrated a clear distinction between specific binding of MPT64 and non-specific  
538 binding of HSA not exceeding 6%  $R_{ct}$  change. The signal after stabilization was used as a reference

539 to calculate the  $R_{ct}$  change during MPT64 detection. A concentration dependent response was ob-  
540 tained with a gradual increase in  $R_{ct}$  signal up to 50 nM MPT64 demonstrating a good response  
541 down to concentrations of 49 pM with the limit of detection (LOD) of 81 pM for MPT64 detection  
542 (**Fig. 6**). The smallest protein concentration tested was 12 pM. Hence, at concentrations of 12 pM  
543 and 24 pM the signal change was negligible. The LOD was calculated using the formula  $LOD =$   
544  $mean_{blank} + 1.645(SD_{blank}) + 1.645(SD_{low\ concentration\ sample})$ , where SD is standard deviation  
545 (Armbruster and Pry, 2008; Armbruster et al., 1994). The signal for diluted serum alone remained  
546 unchanged throughout the experiment. The surface of the sensor started to saturate at around 50  
547 nM of MPT64, similarly to what was observed with the measurements after incubation in SELEX  
548 buffer. The  $R_{ct}$  change gives slightly higher response after incubation with respective concentra-  
549 tions of MPT64 in diluted serum compared to those diluted in SELEX buffer (Supplementary In-  
550 formation, **Fig. S8**). We assume that the composition of serum could promote the formation of  
551 MPT64 multimers. MPT64 can form multimeric structures, tetrameric structures to be exact, in  
552 isolates with subunits being covalently connected via disulphide bonds (Chu and Yuann, 2011).  
553 Therefore, instead of having one MPT64 on the electrode surface it was estimated that tetrameric  
554 MPT64 could be attached onto the aptamer modified electrode. A slight increase in  $R_{ct}$  in serum  
555 measurements could be as the result of an attachment of MPT64 tetramers to the aptasensor sur-  
556 face. MPT64 forms tetrameric structures and not agglomerates, hence, the attachment of MPT64  
557 tetramers to the aptasensor surface could not form a non-specific adsorption. The strong covalent  
558 bond of MPT64 tetramers cannot be removed by sufficient rinsing, unless by adding a detergent  
559 (urea or DTT) to an electrode surface. Since the aptamer was attached onto the electrode surface  
560 via such S-S bond, the addition of detergent to an electrode surface could detach the aptamer from  
561 the electrode surface. The rinsing of the electrode was done sufficiently, and hence, we believe  
562 that the interaction between the aptamer and the MPT64 tetramers are specific.

563 We also performed measurements in less diluted serum (1/10 dilution), which gave a much  
564 higher LOD (12.73 nM) (Supplementary Information, **Fig. S7**). Since there was a pool of other  
565 proteins and molecular agglomerates present in less diluted serum, the chances for MPT64 multi-  
566 mer to move freely and reach the aptasensor surface was limited thereby giving a lower signal  
567 change in the charge transfer resistance. During cellular infection with *Mtb*, mycobacterial prod-  
568 ucts including the MPT64 biomarker incorporates into host cell exosomes, 100 nm vesicles which  
569 are then released into the biological fluid (Giri et al., 2010; Mehaffy et al., 2017). The current

570 clinical diagnostic level of MPT64 protein in isolated exosomes from the serum is at a sub-nano-  
571 molar range (Mehaffy et al., 2017). Hence, the detection limit of the current aptasensor is in the  
572 range of detecting MPT64 in clinical samples and therefore, it could be further adopted as one of  
573 the rapid MPT64 detection methods.

574

#### 575 **4. Conclusion**

576 In this study, strategies for the optimization of an EIS aptamer based sensor for the detection of  
577 *Mtb* secreted antigen MPT64 was investigated. It was concluded that the combination of the long  
578 linker aptamer/MCH was optimal for the sensitive detection of MPT64. The aptamer was specific  
579 to MPT64 as shown by EIS and contact angle measurement experiments. BioFET results con-  
580 firmed the importance of the combination of the long linker aptamer/MCH. The aptamer surface  
581 density of 1/100 ratio provided the maximum impedimetric signal compared to other ratios studied.  
582 The importance of the buffer was also established for MPT64 binding and the SELEX buffer, in  
583 which aptamers were synthesized, was found to be the optimal buffer to conduct the measure-  
584 ments. The aptasensor was tested with spiked human serum and evaluated in dose response anal-  
585 ysis. The measurements were reproducible with a LOD of 81 pM, which is in the range of detecting  
586 MPT64 in clinical samples. The current study showed a significant reduction in detection time  
587 compared to traditional detection methods by reducing time from several hours and/or days to 30  
588 min. Moreover, the aptasensor in this study used a relatively simple surface chemistry based on  
589 co-immobilization of both aptamer and co-adsorbent at the same. The proposed surface chemistry  
590 can be further studied and utilized for the development of a sensitive aptamer-based detection of  
591 MPT64 antigen.

592

#### 593 **5. Acknowledgments**

594 We would like to acknowledge Sunil K. Arya, Pavel Zhuravski and other members of the Bio-  
595 sensor Research Laboratory at the University of Bath for their help, advice and guidance. This  
596 work was supported by the British Council through a Newton-AI Farabi Fund researcher links  
597 travel grant (grant reference: 216423762).

598

#### 599 **Appendix**

600 Supplementary Information.

601

602 **Author Contributions**

603 The manuscript was written through contributions of all authors. All authors have given ap-  
604 proval to the final version of the manuscript.

605

606

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721 **Figure legends**

722 **Fig. 1.** An aptasensor surface modification based on different chemistries applied onto the gold  
723 surface of an electrode; (a) short linker DNA aptamer/MCH; (b) long linker DNA aptamer/EG; (c)  
724 long linker DNA aptamer/MCH. Not to scale.

725  
726 **Fig. 2.** Target MPT64 detection using BioFET. (a) A typical  $I_D/V_{GS}$  characteristic curve for an  
727 electrode before (solid line) and after (dashed line) MPT64 binding event based on long linker  
728 aptamer/MCH complex. (b)  $V_{GS}$  changes on the surface of the electrode upon incubation with dif-  
729 ferent MPT64 concentrations in SELEX buffer based on short linker aptamer/MCH (1/100 ratio),  
730 long linker aptamer/EG (1/100 ratio) and long linker aptamer/MCH (1/100 ratio). All measure-  
731 ments were repeated on three different electrodes.

732  
733 **Fig. 3.** (a) Aptamer molecule density distribution on the electrode surface vs. aptamer/MCH molar  
734 fraction in solution. The measurements were based on the resulting charge change in the absence  
735 and presence of 100  $\mu\text{M}$  hexaammineruthenium (III) chloride ( $\text{Ru}(\text{NH}_3)_6^{3+}$ ) in 10 mM Tris-HCl  
736 pH 7.4. (b) Change in charge transfer resistance ( $R_{ct}$ ) of an electrode measured in 30 min intervals  
737 at different MPT64 concentrations binding at different aptamer/MCH ratios. The measurements  
738 were recorded in SELEX buffer containing 2 mM ferro/ferricyanide  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox couple.  
739 Error bars show the mean and spread for at least three measurements from different electrodes.

740  
741 **Fig. 4.** (a) A schematic overview of the gold electrode surface modification with a long linker  
742 DNA aptamer/MCH followed by the target MPT64 capturing event on the functionalized surface  
743 of the gold electrode; (b) A representative Nyquist plot ( $-Z''$  vs.  $Z'$ ) for an electrode with a bare  
744 surface, immobilized with the long linker DNA aptamer/MCH before and after interaction with  
745 MPT64 for 30 min. Calculated fitted values to the equivalent circuit are presented as dashed lines.  
746 (c) A concentration dependent curve for different MPT64 concentrations using a long linker DNA  
747 aptamer/MCH at a ratio of 1/100 relative to blank measurements from the EIS signal measured in  
748 SELEX buffer; (d) a Nyquist plot for different MPT64 concentrations (in nM) using a long linker  
749 DNA aptamer/MCH in a ratio of 1/100 (experimentally obtained values are shown as dots; fitted  
750 values are shown as dashed lines); Inset (for b and d): Randles equivalent circuit, where  $R_s$  is the  
751 solution resistance,  $R_{ct}$  is the charge transfer resistance,  $CPE$  is the constant phase element, and  $W$

752 is the Warburg element. Error bars show the mean and spread for at least three measurements from  
753 different electrodes.

754  
755 **Fig. 5.** Specificity study of the electrochemical aptasensor for the target MPT64 detection along  
756 with HSA, CEA, and PSA detection at different concentrations based on a long linker DNA ap-  
757 tamer/MCH surface chemistry at an optimized ratio of 1/100. Change in charge transfer resistance  
758 ( $R_{ct}$ ) of an electrode measured in 30 min intervals in SELEX buffer containing 2 mM ferro/ferricy-  
759 anide  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox couple. Error bars show the mean and spread for at least three meas-  
760 urements from different electrodes.

761  
762 **Fig. 6.** (a) A representative Nyquist plot ( $-Z''$  vs.  $Z'$ ) for an electrode immobilized with a long linker  
763 DNA aptamer/MCH in a buffer and in the serum. (b) Change in the charge transfer resistance ( $R_{ct}$ )  
764 of an electrode measured in 30 min intervals at different MPT64 concentrations 1/100 diluted se-  
765 rum samples using EIS. Measurements were recorded in SELEX measurement buffer containing  
766 2 mM ferro/ferricyanide  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox couple. Black squares represent values obtained from  
767 incubation with different MPT64 concentrations diluted in serum. Dashed tread line represents  
768 repetitive measurements of serum sample without MPT64. Error bars show the mean and spread  
769 for at least three measurements from different electrodes.

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772 **Table titles**

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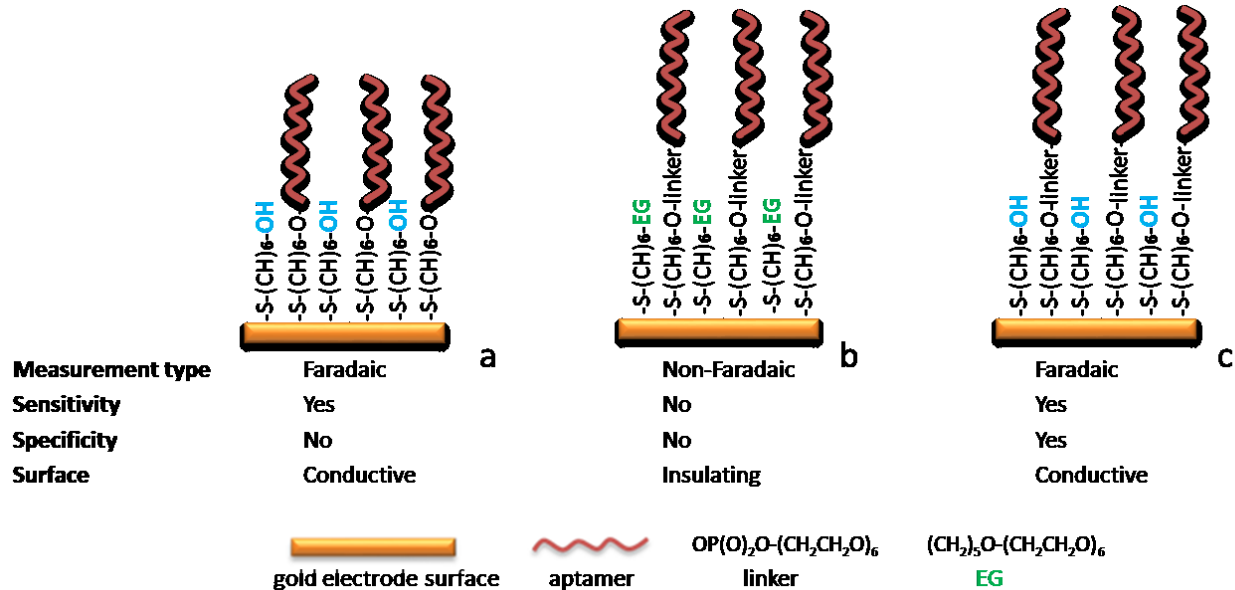
774 **Table 1.** Overview of TB detection methods described in the literature.

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776 **Table 2.** Aptamer molecule density distribution on the electrode surface vs. aptamer/MCH molar  
777 fraction in solution.

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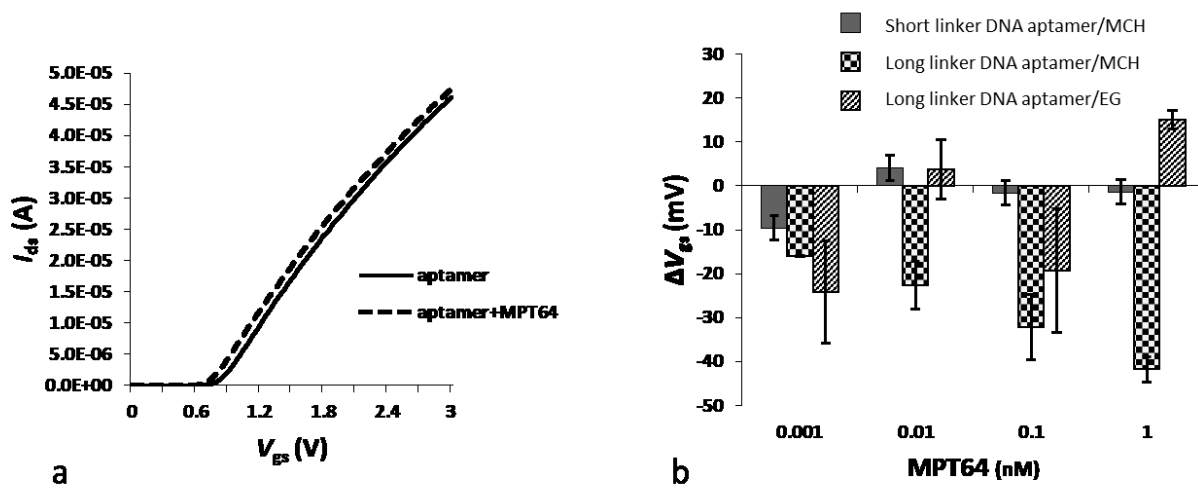
Fig. 1.



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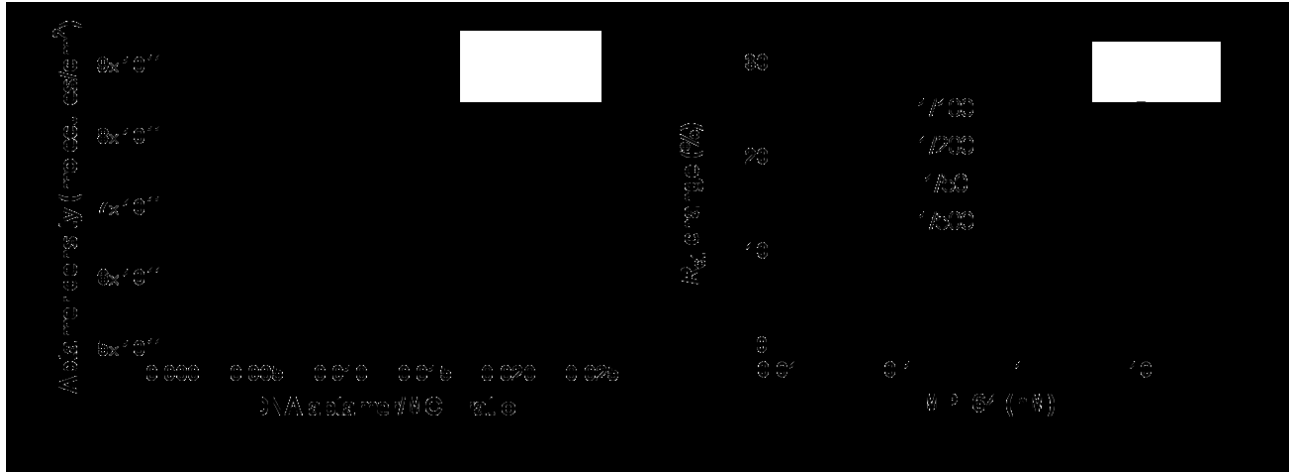
Fig. 2.



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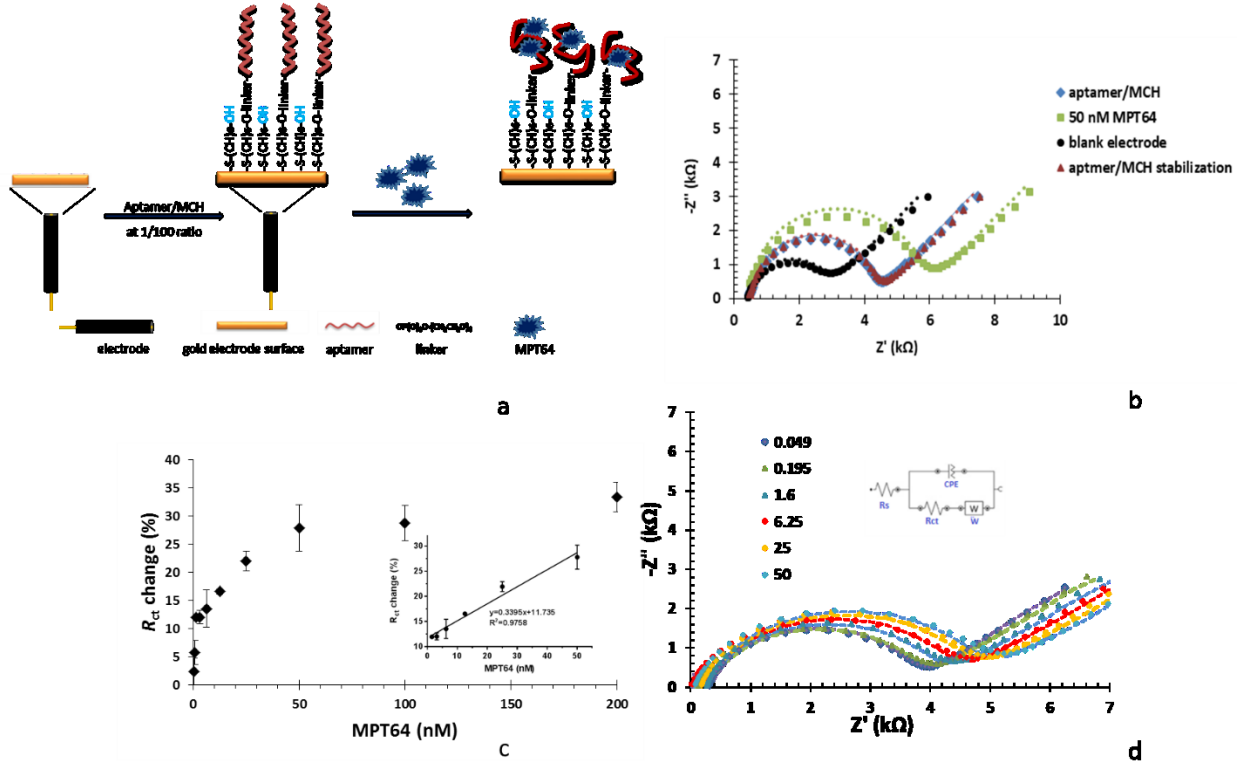
Fig. 3.



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Fig. 4.

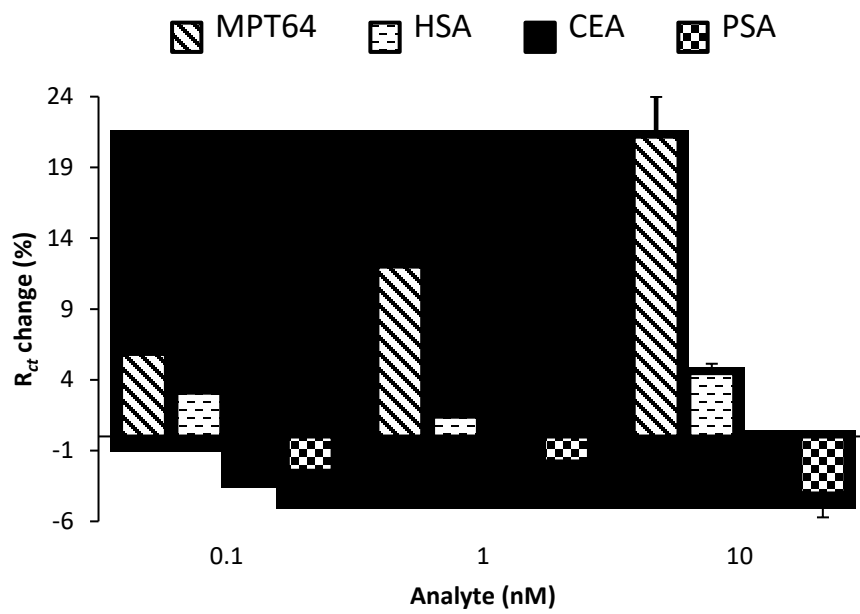


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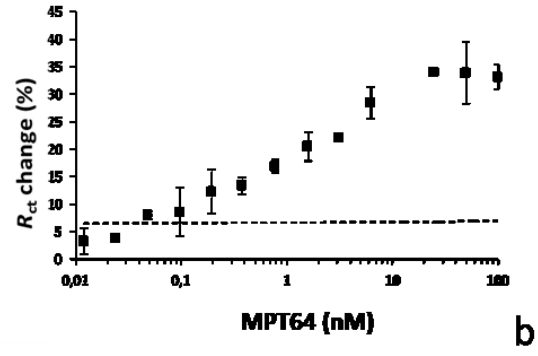
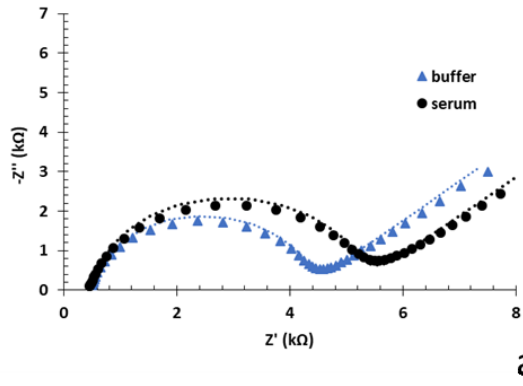
Fig. 5.



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Fig. 6.



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Type of method	Sub-division of type of method	Type of technique	Recognition molecule	Disadvantage	Advantage	Reference
Smear microscopy *	Acid-fast bacilli smear microscopy	Sputum smear microscopy	Cell staining	Low sensitivity, not specific to <i>Mtb</i>	Good in poor resource settings	(Parsons et al., 2011)
	Automated	Bactec MGIT 960	Bacterial growth	Sophisticated instrument, contamination, disposal of radioactive material	Decrease in numbers of false positive results	(Tortoli et al., 1999)
Culture method *	Automated	Mb/Bact system	Bacterial growth	Sophisticated instrument, contamination, disposal of radioactive material	Decrease in numbers of false positive results	(Díaz-Infantes et al., 2000)
	Automated	Esp culture system	Bacterial growth	Sophisticated instrument, contamination, disposal of radioactive material	Decrease in numbers of false positive results	(Bergmann and Woods, 1998)
	Semi-automated	Bactec 460	Bacterial growth	Sophisticated instrument, contamination, disposal of radioactive material	Decrease in numbers of false positive results	(Aggarwal et al., 2008)
Blood sample analysis based method	Bacterial detection	Bactec Myco/F Lytic Blood Culture	Bacterial growth	Sophisticated instrument, contamination, disposal of radioactive material	Decrease in numbers of false positive results	(Waite and Woods, 1998)
	Mediated interferon gamma (IFNg) detection	T-SPOTTB test	Antibody	No discrimination of latent TB from active	Response to multiple antigens and discrimination between <i>Mtb</i> and environmental mycobacteria	(Herrera et al., 2011; Pai et al., 2014; Richeldi, 2006)
	Mediated interferon gamma (IFNg) detection	QuantiFeron TB	Antibody	No discrimination of latent TB from active	Response to multiple antigens and discrimination between <i>Mtb</i> and environmental mycobacteria	(Sultan et al., 2013)

Molecular method	Nucleic acid amplification test (PCR, RT-PCR)	Amplification of TB signature genes	DNA hybridization	Sophisticated instrument and expensive reagents	Information of size and sequence of a gene and drug resistance can be obtained	(Kiraz et al., 2010; Russo et al., 2006; Sambarey et al., 2017; Sharma et al., 2012;)
		Xpert MTB/RIF assay *	DNA hybridization	Sophisticated instrument and expensive reagents	Information of size and sequence of a gene and drug resistance can be obtained	(Marlowe et al., 2011)
		Genotype MTBDR plus	DNA hybridization	Sophisticated instrument and expensive reagents	Information of size and sequence of a gene and drug resistance can be obtained	(Hillemann et al., 2007)
		DNA hybridization (microarrays)	DNA hybridization	Sophisticated instrument and expensive reagents	Information of size and sequence of a gene and drug resistance can be obtained	(Talaat et al., 2002)
	DNA sequencing	Whole genome sequencing	DNA hybridization	Sophisticated instrument and expensive reagents	Information of size and sequence of a gene and drug resistance can be obtained	(Lee and Behr, 2016; Walker et al., 2015)
		Specific gene sequencing	DNA hybridization	Sophisticated instrument and expensive reagents	Information of size and sequence of a gene and drug resistance can be obtained	(Zhang et al., 2013)
Skin patch	Tuberculin skin test (TST)		Antibody	Follow-up visit required; wide range of sensitivities and specificities	No sample preparation/treatment required	(Chaudhary et al., 2010)
Immune-based method	Immunochromatographic	One-Step TB Test Kit; Tell Me Fast <i>MTB</i> IgG/IgM Test Device; Capilia TB; SD Bioline TB AG Mpt64; SD TB IgG/IgM; BD MGIT	Antibody	No discrimination between <i>MTB</i> species	Mutation or deletion of the target gene can result in false negatives	(Ngeow et al., 2011; Shen et al., 2011; Toiher et al., 2011)

Electro-chemical detection	Differential pulse voltammetry (DPV)	Electropolymerised Poly(3,4-ethylenedioxythiophene) doped with carbon nanotubes	aptamer 77 nucleotides	Relatively complex surface chemistry. The sensor needs to be validated in real TB samples.	Detection limit within clinical detection range	(Thakur et al., 2017b)
	DPV	Sandwich scheme based on: gold nanoparticles decorated with fullerene-doped polyaniline	aptamer 35 nucleotides	Relatively complex surface chemistry. The sensor needs to be validated in real TB samples.	Detection limit within clinical detection range	(Bai et al., 2017)
	DPV	Graphene modified iron-oxide chitosan hybrid nanocomposite film deposited on fluorine tin oxide	aptamer 77 nucleotides	Relatively complex surface chemistry. The sensor needs to be validated in real TB samples.	Detection limit within clinical detection range	(Thakur et al., 2017a)
	EIS	IDM bare gold electrode with MCH as co-adsorbent	aptamer 40 nucleotides	The sensor needs to be validated with real TB samples	Simple surface chemistry, detection limit within clinical detection range	This work

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811 \* - routinely used assays in anti-tuberculosis dispensaries approved by WHO.

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Table 2

<b>Aptamer/MCH ratio</b>	<b>Mean of measurements from three electrodes (molecules/cm<sup>2</sup>)</b>
1/50 (0.002%)	$6.50 \times 10^{11}$
1/100 (0.005%)	$7.34 \times 10^{11}$
1/200 (0.01%)	$7.66 \times 10^{11}$
1/500 (0.02%)	$7.91 \times 10^{11}$

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