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1                   **Guided mode resonance sensor for the parallel**  
2                   **detection of multiple protein biomarkers in human**  
3                   **urine with high sensitivity**

4  
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18  
19   Keywords: immunosensor, optical biosensor, protein biomarkers, guided mode resonance,  
20   polyethylene glycol, urine matrix.

29 **Abstract**

30 The rising cost of global healthcare provision and new approaches to managing disease are driving  
31 the development of low-cost biosensing modalities, such as label-free photonic methods based on  
32 dielectric resonances. Here, we use the combined sensing and imaging capability of a guided mode  
33 resonance (GMR) sensor to detect multiple biomarkers (troponin, procalcitonin and C-Reactive  
34 Protein) in parallel in undiluted urine samples. A key requirement of such a biosensor is the simple  
35 and direct functionalization with suitable antibodies to ensure the disease-specific detection of  
36 protein biomarkers. Here, antibodies were immobilized using a succinimidyl-[(N-  
37 maleimidopropionamido)-hexaethyleneglycol] ester (SM(PEG)<sub>6</sub>) spacer. The polyethylene glycol  
38 (PEG) chemistry enables low detection limits of 10 pg mL<sup>-1</sup> or better for all protein biomarkers,  
39 while minimizing non-specific binding compared to more commonly used strategies such as (3-  
40 Aminopropyl)triethoxysilane (APTES) or dextran. Our approach supports the vision of a simple  
41 yet highly sensitive diagnostic platform that could be used for pre-screening patients for a wide  
42 range of diseases at point-of-care, thereby relieving the pressure on overstretched healthcare  
43 services.

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## 50        **1. Introduction**

51    Early recognition and targeted treatment of disease is an essential element of healthcare provision.  
52    Being able to detect multiple biomarkers in a single test is particularly desirable, as it allows for a  
53    more accurate and personalised diagnosis or for screening for a wide range of diseases in a single  
54    test. Conducting such a test in a clinical matrix is essential and doing so in urine is particularly  
55    desirable as the sample can be collected non-invasively, which is preferred by patients. The  
56    challenge of using urine as a sample matrix is that the concentration of biomarkers is typically low;  
57    the physiological concentration of many proteins is 3-4 orders of magnitude lower in urine than in  
58    blood plasma (Eamudomkarn et al., 2018), which presents a major challenge to the sensing  
59    modality. For example, the cardiac biomarker troponin needs to be detected at levels of 10-40 pg  
60    mL<sup>-1</sup> in urine for the sensor to be clinically relevant (Tanislav et al., 2016; Upasham et al., 2018).

61    Various sensor technologies have been developed to meet these demands, including  
62    electrochemical, calorimetric, piezoelectric, and optical biosensors (Kazemi-Darsanaki et al., 2013;  
63    Kenaan et al., 2016; Thakur and Ragavan, 2013; Thévenot et al., 2001). Biosensors based on optical  
64    transduction are particularly attractive as they offer high sensitivity, contact-free and simultaneous  
65    detection of multiple biomarkers. Furthermore, the refractive index sensitivity of photonic  
66    resonances can be exploited to enable label-free biomarker detection, further simplifying the  
67    diagnostic procedure. The diagnostic potential of resonant photonic sensors, such as those based  
68    on surface plasmon resonance (SPR), microring resonance or guided mode resonance (GMR) has  
69    already been demonstrated. While waveguide-based sensors such as microrings (Luchansky et al.,  
70    2010) and bimodal waveguides (Herranz et al., 2017) are the most sensitive amongst these, they  
71    require accurate alignment strategies in order to couple the light into the waveguide which makes  
72    them difficult to implement in low-cost, point-of-care solutions. In contrast, the leaky nature of

73 GMR-based biosensors means light can be coupled easily into the grating using a simple collimated  
74 beam. The key question is then whether the intrinsically low quality factor of the leaky mode  
75 approach prevents a sensor from achieving the high sensitivity required to detect disease  
76 biomarkers in urine?

77 The GMR sensing modality was first proposed by Wang and Magnusson (Wang and Magnusson,  
78 1993), and demonstrated experimentally as fiber endface biosensor by Wawro (Wawro et al., 2000).  
79 Later, Cunningham et al. showed that the grating structure can be fabricated inexpensively by  
80 replica moulding or nanoimprint techniques (Cunningham, 2010). Recently, we introduced the  
81 chirped GMR approach (Triggs et al., 2017). The chirp translates spectral information into spatial  
82 position, so the refractive index change caused by molecular binding can be detected simply by  
83 imaging the spatial position of the optical resonance. This means that the bulky and expensive  
84 spectrometer typically required for monitoring the shift in optical resonance can be replaced with  
85 a simple, low cost camera. Here, we take this imaging capability one step further and demonstrate  
86 that multiple sensing areas can be monitored in parallel, thereby adding multiplexing capability  
87 (Fig. 1).

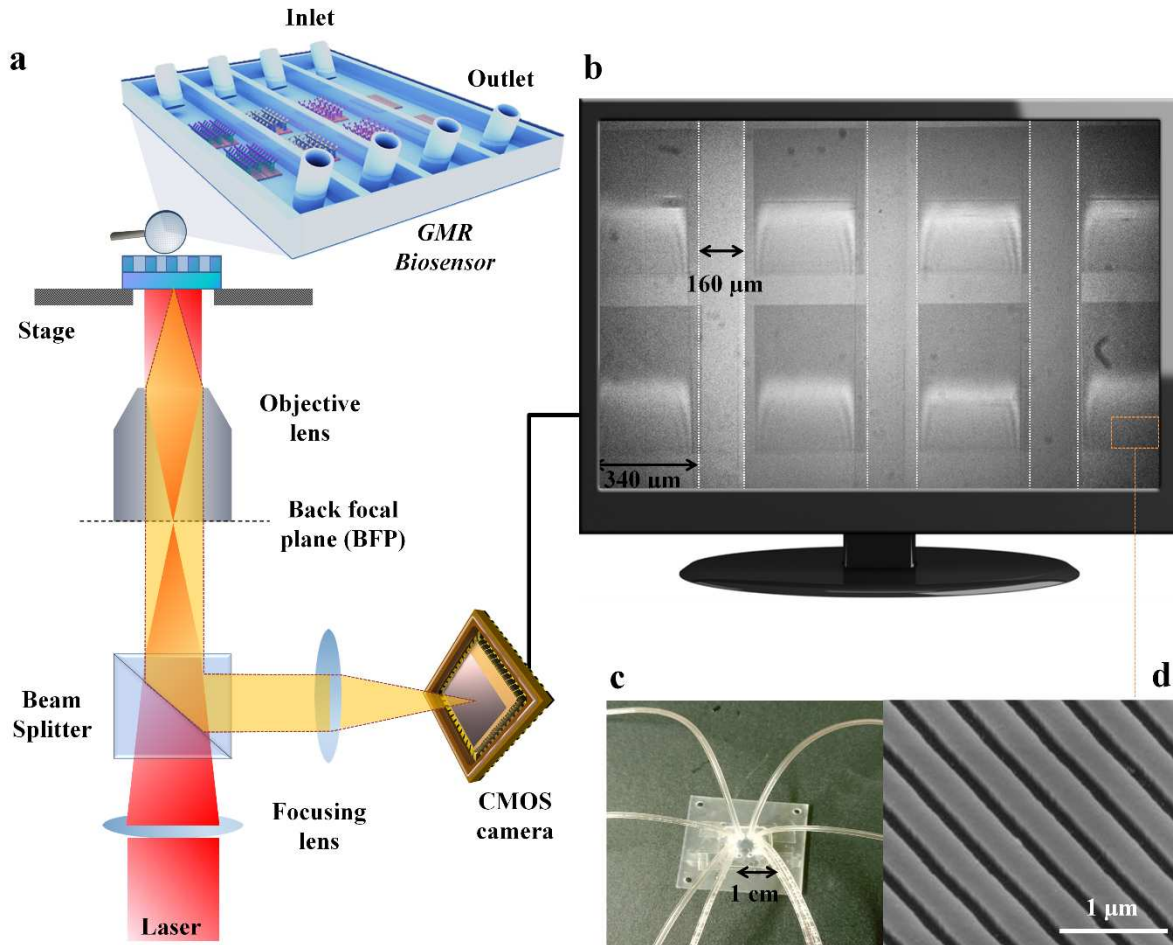
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93 **Fig. 1. Multiplexing capability of a chirped GMR sensor.** a) Schematic diagram of the measurement  
 94 setup showing the multiplexed chirped GMR consisting of four independent measurement channels. Three  
 95 channels are functionalized with biomarker-specific antibodies, here C-reactive protein (CRP), troponin  
 96 (TNNT1) and procalcitonin (PCT). The fourth channel is unfunctionalized and used as a reference to account  
 97 for systemic drifts e.g. due to temperature. b) Field of view of the camera showing all 4 channels. Each  
 98 channel contains two GMRs to provide redundancy and increase fidelity. c) The microfluidic channels are  
 99 made of PDMS and are connected separately. d) SEM micrograph of the grating made in silicon nitride.

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102 A GMR is a refractive index sensor that requires surface functionalization with a capture molecule,  
 103 such as an antibody, to gain specificity. Limits of detection achieved so far using a functionalized  
 104 chirped-GMR sensor are in the  $\text{ng mL}^{-1}$  range, which is comparable with other leaky-mode

105 modalities, e.g. plasmonic nanoholes ( $145 \text{ pg mL}^{-1}$ ) (Li et al., 2017). Nevertheless, in order to be  
106 truly competitive with conventional diagnostics based on the enzyme-linked immunoassay  
107 (ELISA), and to provide clinically relevant sensitivity, the sensor needs to demonstrate 1-10 pg  
108  $\text{mL}^{-1}$  sensitivity. Here, we demonstrate performance at this level by introducing an improved  
109 functionalization protocol.

110 The key requirement for any functionalization protocol is high affinity binding to a particular target  
111 molecule, coupled with the minimization of non-specific binding events which would otherwise  
112 reduce the detection specificity. The latter requirement of non-specific binding is often overlooked;  
113 many studies in the literature have been conducted with laboratory dilutions that avoid non-specific  
114 binding simply by the absence of competing agents instead of by optimizing the protocol for real  
115 sample matrices such as undiluted urine or blood. Furthermore, many protocols are carefully  
116 optimized for a specific antigen/antibody pair. However, achieving high performance for  
117 multiplexed detection without optimizing each assay is still a challenge.

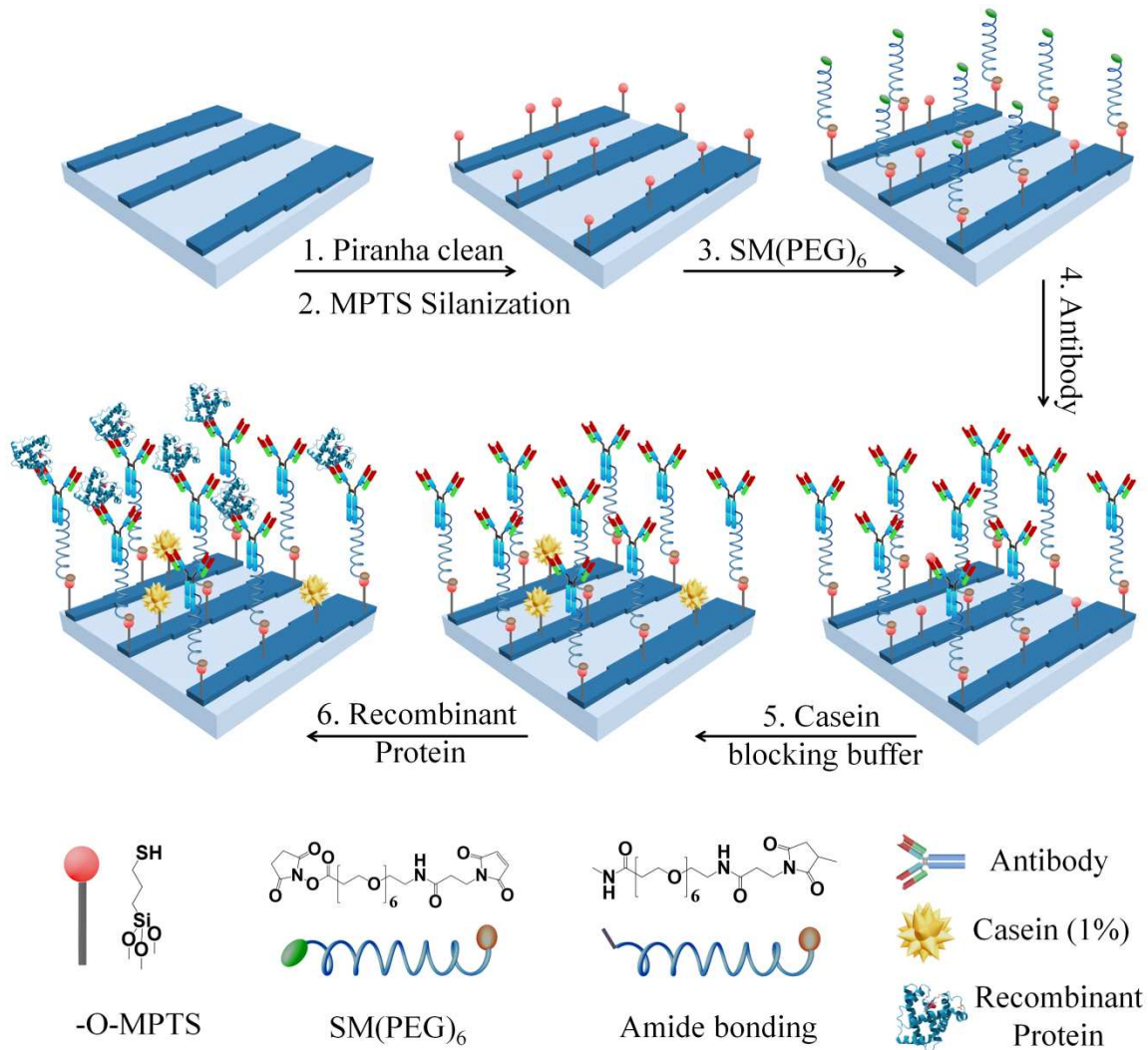
118 The functionalization of dielectric or silicon surfaces used in photonic biosensors typically employ  
119 silane chemistries to render the surface reactive against carboxylate or amine groups exposed on  
120 an antibody surface (Vashist, 2012). For example, (3-Aminopropyl)triethoxysilane (APTES) is  
121 commonly used to generate primary amine groups on the sensor surface to which the antibodies  
122 are crosslinked via exposed carboxylate groups using the EDC/NHS chemistry (1-Ethyl-3-(3-  
123 dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide, resulting in the formation of a stable  
124 amide bond. While simple and inexpensive, the amine coupling chemistry can lead to  
125 multimerization of the activated antibodies that may mask its binding sites and introduce  
126 conformational stress (Dixit et al., 2011; Stefansson et al., 2012), and lead to reduced specificity  
127 due to non-specific binding to the free amine groups that have not been occupied by an antibody.

128 Moreover, the chain length of APTES is short resulting in steric hindrance that impedes antigen  
129 binding (Kim and Herr, 2013; Makaraviciute and Ramanaviciene, 2013). A longer, flexible spacer  
130 consisting of a hydrophilic, anti-fouling polymer is desirable in order to enable higher density  
131 immobilization of antibodies with steric freedom (Jönsson et al., 2008; Yakovleva et al., 2003). A  
132 common example of such a spacer is dextran, which provides a large volume of antibody binding  
133 sites because of its porous structure and its large molecular weight (Kim and Herr, 2013; Lee et al.,  
134 2013). Although the dextran matrix provides the sensor surface with a large number of binding  
135 sites, many of these sites can also cause significant non-specific binding, which is clearly  
136 undesirable.

137 Here, we exploit the commercially available SM(PEG)<sub>6</sub> spacer (succinimidyl-[(N-  
138 maleimidopropionamido)-hexaethyleneglycol] ester) as a crosslinker between the sensor surface  
139 and the antibody. PEG has previously been adopted to reduce steric hindrance, improve water  
140 solubility and reduce aggregation (Kim and Herr, 2013; Li et al., 2016; Nagasaki et al., 2007;  
141 Pochechueva et al., 2014; Weimer et al., 2000; Wen et al., 2009; Yuan et al., 2014). SM(PEG)<sub>6</sub> is  
142 a sulfhydryl and amine reactive heterofunctional polyethylene glycol (PEG) with N-  
143 hydroxysuccinimide (NHS) ester and maleimide groups at the termini. Maleimide terminated PEG  
144 is thiol-reactive which is used in our binding assay for bioconjugation with the sensor surface that  
145 has been thiolated using 3-Mercaptopropyl)trimethoxysilane (MPTS). This results in a surface  
146 functionalized with a monolayer of NHS esters which can form a covalent bond with free amines  
147 exposed on the antibody surface. We also note that the physical thickness of the SM(PEG)<sub>6</sub>  
148 monolayer is < 3 nm, which is much shorter than the evanescent tail of the GMR mode of >100



149 nm (Drayton et al., 2019). We thus do not expect the SM(PEG)<sub>6</sub> spacer to adversely affect the  
 150 sensitivity of the GMR. The functionalization protocol is illustrated in Fig. 2.



151 **Fig. 2. Functionalization protocol using PEG as a spacer on a silicon nitride GMR.** (1)  
 152 Hydroxyl groups (OH) are introduced to the surface by piranha treatment. (2) Sulfhydryl groups  
 153 are generated by (3-Mercaptopropyl)trimethoxysilane (MPTS) silanization for 7h. (3) SM(PEG)<sub>6</sub>  
 154 crosslinkers are introduced to the sulfhydryl groups via the maleimide groups after incubation  
 155 overnight in DMSO. (4) Antibody is introduced and immobilized on the PEGylated surface via its  
 primary amine group to form an amide bond after 60 min of incubation. (5) Casein blocking buffer  
 (1%) is added for ~30 min to block non-specific binding sites. (6) The Recombinant protein is  
 added to the antibody sites at  $T = 37^{\circ}\text{C}$ . Steps 2-6 are performed inside the microfluidic channel.

## 156 2. Experimental section

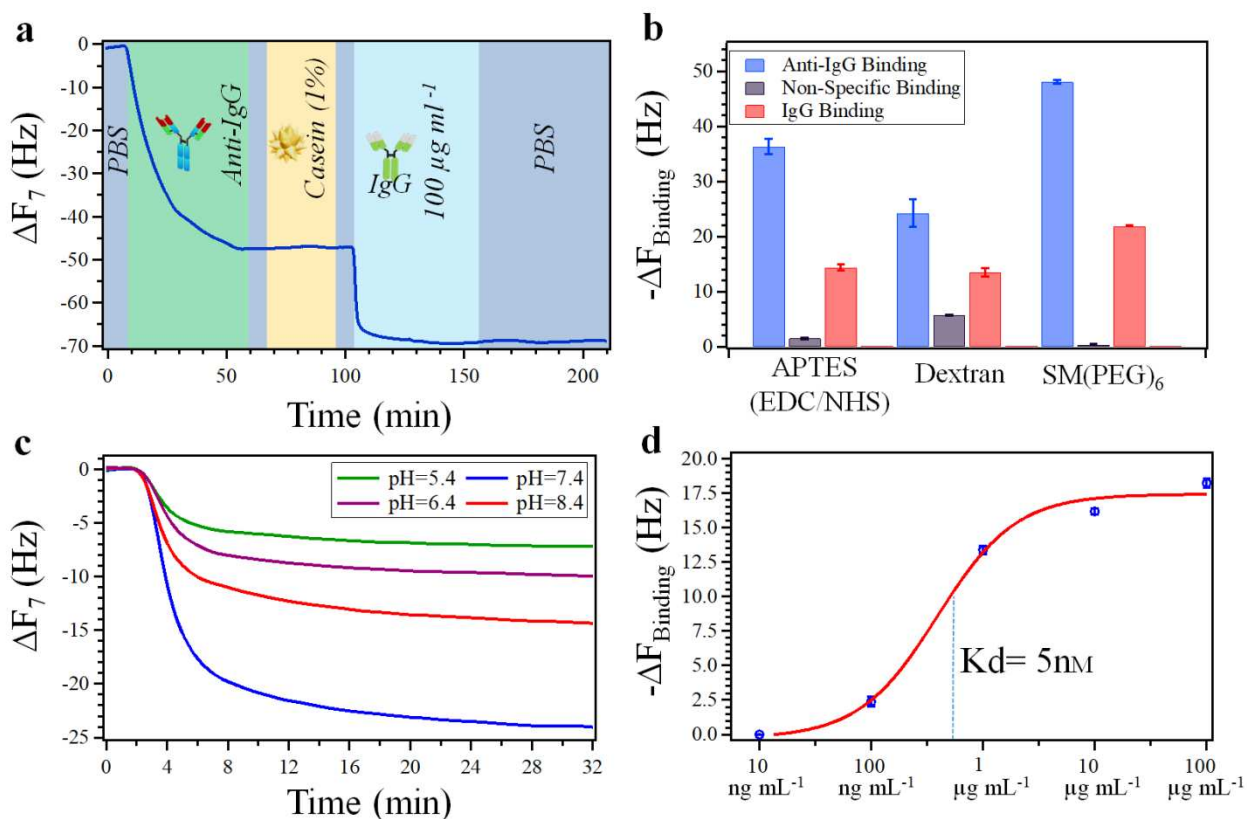
157 Sub-sections regarding materials, chirped GMR fabrication, microfluidics, channels fabrication,  
158 and functionalization protocols including EDC/Sulfo-NHS, SM(PEG)<sub>6</sub>, and Dextran chemistry are  
159 provided in the supplementary information file.

## 160 3. Results

### 161 3.1. Quartz crystal microbalance with dissipation (QCM-D) analysis

162 We first used a quartz crystal microbalance with dissipation (QCM-D) using silicon dioxide quartz  
163 sensor to optimize the functionalization protocol using an antibody against Immunoglobulin G (IgG)  
164 (anti-IgG) as an exemplar (see experimental methods for detail). QCM-D is a well-established  
165 reference tool that provides quantitative data, so is ideally suited for process development. A clear  
166 binding curve is observed as the anti-IgG immobilizes on the PEG spacer Fig. 3a. Upon saturation,  
167 the surface is washed with PBS to remove unbound antibodies, followed by flushing with casein  
168 buffer (1%) to block any remaining non-specific binding sites. We observe negligible binding of  
169 casein suggesting that the PEG forms a densely packed, anti-fouling monolayer. Finally, IgG is  
170 introduced into the QCMD flow cell leading to a clear binding curve. For comparison, the protocol  
171 was repeated for surfaces functionalized with APTES and dextran (see methods section and Fig.  
172 S2a and Fig. S2b for more detail). The specific and non-specific binding for these surfaces in  
173 comparison to the PEG functionalized sensor is quantified in Fig. 3b and Table S1. The shift in  
174 resonant frequency upon exposure to anti-IgG is highest for PEG, indicating a greater density of  
175 surface immobilized antibodies. This is mirrored by the IgG binding (red bars). Importantly, the  
176 amount of non-specific binding is smallest for the PEG functionalized surface (using casein as an  
177 indicator for non-specific binding). Overall, PEG offers the highest *specific* and the lowest *non-*  
178 *specific* binding of the three protocols, along with higher number of antibodies immobilized on the

179 surface (Fig. S2c and Fig. S2d), which is likely due to the minimization of steric hindrance coupled  
 180 with the anti-fouling properties inherent to PEG monolayers. This combination of properties is  
 181 critical for highly sensitive biomarker detection in a clinical matrix.



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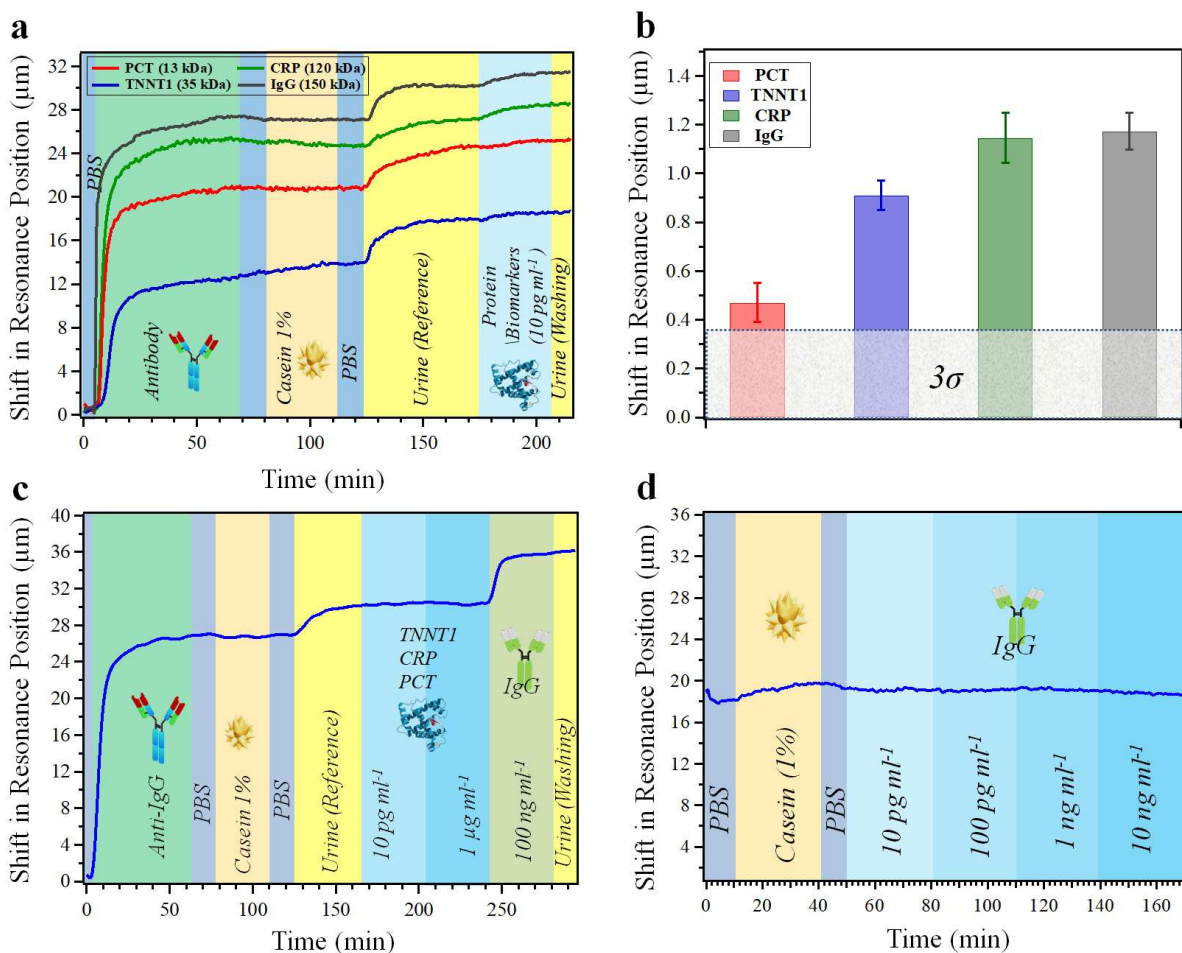
**Fig. 3. Quartz crystal microbalance with dissipation (QCM-D) analysis.** (a) IgG binding assay employing SM(PEG)<sub>6</sub> at pH = 7.4. (b) Comparison of the different functionalization protocols (APTES, Dextran and PEG) in terms of non-specific binding and in terms of frequency shift upon binding to IgG. Each point represents the mean ±SD of three replicates. (c) pH dependence on IgG binding. (d) IgG binding to the PEGylated surface as a function of concentration. Each point represents the mean ±SD of three replicates.

183 Fig. 3c shows the pH-dependence of binding between IgG and surface immobilized anti-IgG for a  
 184 pH range between 5.4 and 8.4, which highlights the best performance at near neutral pH (maximum  
 185 at pH = 7.4), in agreement with the results reported in the literature (Barnes, 1966; Hughes-Jones

186 et al., 1964; Yang et al., 2017). Finally, we examined the binding performance as a function of IgG  
187 concentration at  $pH = 7.4$  for concentrations between  $10 \text{ ng mL}^{-1}$  to  $100 \text{ } \mu\text{g mL}^{-1}$  and observe a  
188 clear binding Langmuir isotherm (Fig. S2d), which allows us to determine a dissociation constant  
189  $K_D$  of  $5 \text{ nM}$  ( $750 \text{ ng mL}^{-1}$ ), in close agreement with published values ( $\sim 4 \text{ nM}$ ) (Kuo and  
190 Lauffenburger, 1993; Strauch et al., 2014).

### 191 **3.2. Chirped guided mode resonance (GMR) analysis**

192 Following the QCMD control experiments, we applied the PEG protocol to the chirped GMR  
193 sensor. The calibration indicates that a  $1 \text{ } \mu\text{m}$  shift in position corresponds to  $1.67 \times 10^{-4}$  refractive  
194 index units (RIU) (Triggs et al., 2017). Given the noise figure of  $0.35 \text{ } \mu\text{m}$  (Fig. S3), this corresponds  
195 to a limit of detection of  $5.8 \times 10^{-5}$  RIU. Note that the results in Fig. 4 were all obtained in undiluted  
196 human urine adjusted to  $pH = 7.4$ . The full measurement sequence is shown in Fig. 4. After  
197 introducing the  $\text{SM(PEG)}_6$  overnight to ensure the maximum coverage (Fig. S4), un-bound  
198 reagents are removed by a dimethyl sulfoxide (DMSO) wash, followed by drying with nitrogen  
199 gas. The surface is then exposed to PBS at  $pH = 7.4$  to establish a baseline. Next, the antibodies  
200 are added (anti-IgG, anti-CRP, anti-PCT and anti-TNNT1) through the independent  
201 polydimethylsiloxane (PDMS) flow channels, followed by the casein blocker. A clear binding  
202 curve is observed associated with immobilization of the antibodies on the sensor surface. Again,  
203 we only observe a negligible shift in resonance following exposure to the casein blocker,



**Fig. 4. Binding assay using PEGylated chirped GMR sensor.** (a) Demonstration of binding assay conducted in urine ( $pH = 7.4$ ), showing the shift in resonance position against time with each assay step. Each channel comprises a single antibody that is challenged with its associated, recombinant antigen. A clear binding curve is observed for each of the 4 proteins at a concentration of  $10 \text{ pg mL}^{-1}$ , i.e. procalcitonin (PCT), C-reactive protein (CRP), troponin (TNNT1), and Immunoglobulin G (IgG). (b) Comparison of the maximum shift observed for the 4 proteins, for a concentration of  $10 \text{ pg mL}^{-1}$ . Each point represents the mean  $\pm$ SD of four replicates. The noise level value ( $3\sigma$ ) is also indicated. (c) Controlled measurement, demonstrating that no binding is observed for mixture of TNNT1, CRP and PCT against an IgG antibody. (d) Similarly, no IgG binding is observed in the absence of a suitable antibody, demonstrating that the curves in (a) are not due to physisorption.

205 functionalization, the four channels were exposed to urine which leads to a shift in resonance due  
206 to the higher refractive index; we see a similar effect in a control experiment conducted with PBS  
207 and urine in the absence of antibody (Fig. S5). Later, we add urine spiked with a recombinant  
208 protein biomarker at a concentration of  $10 \text{ pg mL}^{-1}$  and see clear shifts for all channels. The urine  
209 pH was adjusted by adding a few microliters of a strong base (NaOH) to 45 ml of urine, thus the  
210 dilution factor is negligible. It is worth noting that the binding efficiency of each antigen-antibody  
211 pair depends on the pH of the medium. While it would be ideal to optimize the pH for each channel,  
212 this would complicate sample preparation and would not be possible in a multiplexed format, so  
213 would be undesirable for point-of-care applications. Despite these limitations, our results show an  
214 excellent sensitivity at a single pH-value for all 4 antibody-antigen pairs.

215 IgG immunoassay is conducted in individual channel. We also used a reference channel to  
216 minimize the influence of temperature variations and background noise, by subtracting from the  
217 measurement channel, see supporting information (Fig. S6). Finally, a urine washing step is  
218 performed after the addition of biomarkers. No shift in the resonance position is observed,  
219 indicative of antibody-antigen binding rather than physisorption. Interestingly, these results are  
220 similar and within experimental error to those conducted in PBS rather than urine (Fig. S7), which  
221 again highlights the good surface coverage of the PEG and the suppression of non-specific binding.  
222 As urine typically contains a high level of salts, the ionic strength of the solution may impact on  
223 the weak non-covalent bond between antibody and antigen. We are therefore particularly pleased  
224 to note the high efficiency of the PEG-functionalised surface in terms of anchoring antibodies and  
225 enabling binding to their complementary antigens, even in a matrix of undiluted human urine. Fig.  
226 4b shows the resonant shifts observed for  $10 \text{ pg mL}^{-1}$  of the four protein biomarkers ( $0.47 \text{ } \mu\text{m}$   
227  $\pm 17 \%$ ,  $0.91 \text{ } \mu\text{m} \pm 7 \%$ ,  $1.15 \text{ } \mu\text{m} \pm 9 \%$ , and  $1.17 \text{ } \mu\text{m} \pm 6 \%$  for PCT, TNNT1, CRP, and IgG,

228 respectively). Since the GMR is a refractive index sensor, one would expect that the magnitude of  
229 the resonant shift would increase with molecular weight. This trend is observed qualitatively, i.e.  
230 the smallest molecule (PCT, 13 kDa) produces the smallest shift, and IgG (150 kDa) the largest,  
231 although as expected the difference does not scale quantitatively with mass. Finally, we add urine  
232 spiked with IgG, TNNT1, and PCT at concentrations ranging from 10 pg mL<sup>-1</sup> up to 1 μg mL<sup>-1</sup>,  
233 along with CRP at concentrations ranging from 1 pg mL<sup>-1</sup> up to 1 μg mL<sup>-1</sup> (Fig. S8).

234 Further controlled experiments confirm that we observe specific interactions between the  
235 immobilized antibodies and the associated antigen rather than physisorption. Firstly, we observe  
236 no shift in resonance when a sensor surface functionalized with anti-IGG is challenged with urine  
237 spiked with a mixture of TNNT1, CRP and PCT (Fig. 4c). When the same surface is subsequently  
238 exposed to IgG at 100 ng mL<sup>-1</sup>, we observe a clear binding curve, highlighting that the antibody  
239 binding sites remain active following exposure to other proteins. Secondly, Fig. 4d shows a PEG  
240 functionalized but antibody-free surface exposed to IgG. No significant binding of IgG is observed.  
241 We therefore unambiguously demonstrate that the binding curves observed are due to the formation  
242 of an antibody-antigen complex.

### 243 **3.3. Comparison to previous results**

244 It is interesting to compare our result to competing approaches. Most notably, surface plasmon  
245 resonance (SPR) is considered by many as the gold standard for label-free sensing via photonic  
246 resonances. SPR-based biosensors have been reported that are capable of detecting protein  
247 biomarkers with a detection limit of 460 pg mL<sup>-1</sup> in 1:1 diluted urine (Soler et al., 2016), and 140  
248 pg mL<sup>-1</sup> in PBS (Li et al., 2017). This comparison clearly shows that the GMR technology is highly  
249 competitive, especially in conjunction with the PEG surface functionalization protocol, which

250 offers highly efficient and selective binding of antigens while suppressing non-specific binding.  
251 More importantly, the performance we quote is comparable to the typical detection limit quoted  
252 for an enzyme-linked immunoassay (ELISA), the laboratory gold standard, which is in the low pg  
253 mL<sup>-1</sup> range (Sui et al., 2006). This means that the intrinsically simple and label-free GMR approach  
254 achieves the same performance as a fluorescent label-based ELISA that requires trained laboratory  
255 personnel, which is quite remarkable.

#### 256 **4. Discussion**

257 We have exploited the combined sensing and imaging capability of a chirped guided mode  
258 resonance sensor to demonstrate label-free detection of multiple disease-relevant proteins in  
259 undiluted human urine. Specifically, we have demonstrated the detection of four protein  
260 biomarkers, all at a limit of detection of better than 10 pg mL<sup>-1</sup>. The high performance and ability  
261 to perform multiple tests in parallel is supported by the use of a SM(PEG)<sub>6</sub> spacer layer in the  
262 functionalization protocol which introduces steric freedom and permits the immobilization of  
263 antibodies at high density while minimizing nonspecific binding. We have verified this  
264 performance against comparable protocols using APTES and dextran that are typically used for  
265 dielectric sensor surfaces and have shown improved performance. A comparison against other  
266 photonic sensor modalities is made more difficult by the fact that most published results only refer  
267 to laboratory dilutions, which avoid nonspecific binding by the absence of competing agents, while  
268 our results are achieved in undiluted human urine. Moreover, our sensor and photonic readout  
269 mechanism is intrinsically low-cost and does not require careful alignment or expensive  
270 components, which sets it further apart. In this regard, it is particularly surprising that our label-  
271 free approach now achieves the same pg mL<sup>-1</sup> performance as commercially available enzyme-  
272 linked immunoassays (ELISAs) that use labels and require trained laboratory personnel.



## 273 **5. Conclusion**

274 In conclusion, the parallel detection of 4 clinically relevant biomarkers in human urine, all at  
275 concentrations of 10 pg ml<sup>-1</sup> or below, demonstrates that the guided mode resonance (GMR)  
276 sensing modality, together with a highly efficient PEGylation process for immobilizing antibodies,  
277 offers a favorable combination of properties for the realization of low-cost, high performance  
278 biosensors suitable for evaluating clinical samples. The technology is intrinsically simple, yet it is  
279 essential to demonstrate similar performance as shown here also in a handheld format and in a  
280 clinical setting in order to prove its true value as a point-of-care tool.

## 281 **Conflicts of interest**

282 The authors report no conflicts of interest in this work.

## 283 **Acknowledgment**

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## 290 **Appendix A. Supplementary data**

291 Supplementary data associated with this article can be found in the online version.

292 S1. Experimental section

293 S2. IgG binding assay for QCMD analysis using APTES, dextran, and SM(PEG)<sub>6</sub> protocols

- 294 S3. GMR calibration and data analysis
- 295 S4. Effect of SM(PEG)<sub>6</sub> exposure time on the anti-IgG binding activity
- 296 S5. Effect of refractive index on the resonance position
- 297 S6. Influence of temperature and mechanical noise on the IgG binding assay
- 298 S7. IgG binding assays in PBS
- 299 S8. Proteins binding assays in urine at different concentrations
- 300 Table S1. QCM-D analysis of the different functionalization protocols

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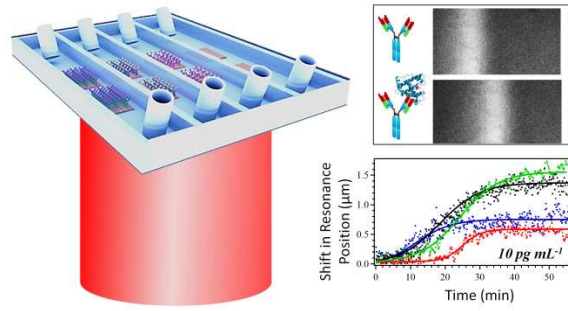
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