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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/148126> since 2017-05-25T17:50:17Z

Published version:

DOI:10.1007/s00216-014-7885-6

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UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

ANALYTICAL AND BIOANALYTICAL CHEMISTRY, 406 (2014), 4841-4849

DOI: 10.1007/s00216-014-7885-6

The definitive version is available at:

La versione definitiva è disponibile alla URL:

<http://link.springer.com/article/10.1007%2Fs00216-014-7885-6>

1 MULTI-ANALYTE HOMOGENOUS IMMUNOASSAY BASED ON QUENCHING OF QUANTUM DOTS BY
2 FUNCTIONALIZED GRAPHENE

3 L. Anfossi^{1*}, P. Calza¹, F. Sordello¹, C. Giovannoli¹, F. Di Nardo¹, C. Passini¹, M.Cerruti², I.Y.
4 Goryacheva³, E.S. Speranskaya³, C. Baggiani¹

5
6 ¹ Department of Chemistry, University of Turin. Via Giuria, 5, I-10125 Turin, Italy

7 ² Materials Engineering, McGill University, 3610 University St., Montreal, QC H3A 0C5, Canada

8 ³ Department of General and Inorganic Chemistry, Chemistry Institute, Saratov State University,
9 Astrakhanskaya 83, 410012 Saratov, Russia

10
11
12 *to whom correspondence should be addressed. Tel: +390116705219, fax: +390116705242. E-mail:
13 laura.anfossi@unito.it

14
15 **Abstract**

16 We propose a homogenous multi-analyte immunoassay based on the quenching of quantum dot
17 (QD) fluorescence by means of graphene. Two QDs with emission maxima at 636 and 607 nm were bound
18 to antibodies selective for mouse or chicken immunoglobulins, respectively, and graphene functionalized
19 with carboxylic moieties was employed to covalently link the respective antigen. The antibody-antigen
20 interaction led graphene close enough to QDs to quench the QD fluorescence by resonance energy
21 transfer. The addition of free antigens that competed with those linked to graphene acted as a “turn on”
22 effect on QD fluorescence. Fluorescence emitted by the two QDs could be recorded simultaneously since
23 the QDs emitted light at different wavelengths while being excited at the same, and proved to be linearly
24 correlated with free antigen concentration. The developed assay allows measuring both antigens over two-
25 three orders of magnitude and showed estimated limits of detection in the nanomolar range. This approach
26 is thus a promising universal strategy to develop homogenous immunoassays for diverse antigens (cells,
27 proteins, low-molecular-mass analytes) in a multi-analyte configuration.

28
29 **Keywords:** immunoassay, antibody, homogeneous assay, quantum dot, graphene, resonance energy
30 transfer, fluorescence quenching

31
32 **Introduction**

33 Quantum dots (QDs) are fluorescent semiconductor nanocrystals with extremely high quantum
34 yields, narrow and tunable emission spectra, wide excitation range, high resistance to photobleaching, and
35 simultaneous excitation of multiple fluorescence colors [1,2]. These exceptional optical properties make

36 QDs very attractive in a number of applications including solar cells [3], LED screens [4] and, recently, labels
37 for biosensing of analytes including DNA, proteins, cells, and small organic molecules [2,5-7]. QDs are
38 typically core/shell inorganic particles with sizes comprised between 2 and 10 nm, often covered with
39 hydrophilic polymeric films for water compatibility and bioconjugation. When acting as biolabels, QDs have
40 been mostly used as donors in biosensors based on the Förster resonance energy transfer (FRET)
41 mechanism [5,8-9]; however, there are also a few examples of QDs used as fluorescent labels for the
42 development of highly-sensitive [10-12], multi-analyte immunoassays [13-15].

43 Just a few years ago, Quian and co-workers showed a simple and elegant fluoroimmunoassay based
44 on the optical properties of QDs and the fluorescence quenching ability of graphene [5]. In this sandwich
45 immunoassay the capture antibody was covalently linked to hydrothermally converted graphene sheets
46 and the reporter antibody was labelled with a QD. Adding the antigen to a solution containing both
47 antibodies gave rise to the formation of a two-site immunocomplex that led the QDs close enough to
48 graphene to determine the transfer of energy and fluorescence quenching, and thus acted as a “turn off”
49 input on QD emission. The quenching efficiency depended on the antigen concentration and was
50 independent over a wide range of distances (tens to one hundred nanometers) between the donor (QD)
51 and the quencher (graphene).

52 In the wake of this pioneering work, Chen and co-workers designed another competitive
53 homogenous immunoassay exploiting the QD-graphene energy transfer, however with a “turn on” model.
54 In this case, QD-labelled antibodies directly interacted with graphene oxide sheets via π - π stacking and
55 hydrogen bonding. If a large antigen such as a human virus bound to the antibody, the distance between
56 the QD and the graphene oxide sheet increased enough to suppress the energy transfer between the QD
57 and graphene oxide, and the QD luminescence was turned on [2]. This “turn on” model showed a higher
58 efficiency and lower background than the “turn off” model. In addition, authors proved the multi-analyte
59 detection potential of their strategy, by using QDs that could be excited at the same excitation wavelength
60 and emitted differently colored fluorescence signals to simultaneously detect two human viruses.
61 Nevertheless, the use of different antibodies by other authors did not give the same results shown by Chen
62 and co-workers [16], and this assay format was limited to the detection of very large antigens, such as cells
63 and pathogens.

64 Based on Chen’s work, Quian et al very recently described a more general fluoroimmunoassay design
65 based on the energy transfer between QD-loaded graphene (QDGNP) and graphene (GNP) [16]. Antibodies
66 are linked to the QDGNP donor through the QD portion of the ensemble, and the proximity between
67 QDGNP and GNP is guaranteed by π - π interactions between GNP and QDGNP sheets. This is a “turn on”
68 assay, where the QD luminescence is turned on when an immunocomplex forms between the labelled
69 antibody and its macromolecular antigen (human IgG), thus pushing QDGNP away from GNP [16]. Although

70 this strategy does not require the antibody to interact with GNP via π - π stacking, which limited the type of
71 antibodies that could be used in Chen's assay [2], it is still constrained by antigen nature and dimension.

72 In a previous work, we functionalized graphene with carboxylate groups on GNP (GNP-COOH) that
73 could act as shape controller i.e. towards TiO₂ nanoplates [17-18]. We propose here an approach to
74 develop a homogenous fluoroimmunoassay based on QD-labelled antibodies and functionalized graphene
75 (GNP-COOH) covalently linked to the antigen as the QD fluorescence quencher. The assay is designed as a
76 competitive immunoassay, in which the free antigen, i.e. the analytical target, and the GNP-linked antigen
77 compete for binding to the QD-labelled antibody. The displacement of GNP-linked antigen from the
78 immunocomplex due to competition with the free antigen determines the restoration of QD luminescence.
79 Therefore, the free antigen determines and modules a "turn on" effect on QD fluorescence emission, as
80 schematically illustrated in Figure 1. The surface modification of GNP [19-23] allows us to covalently
81 conjugate antigens to the quencher thus bringing the QD-labelled antibody close enough to the GNP to
82 permit energy transfer. We show that it is possible to simultaneously determine the concentration of two
83 antigens by using antibodies labelled with QDs that differ in emitted light colour while being excited at the
84 same wavelength. This strategy can be regarded as a truly universal approach, since different antigens
85 (cells, proteins, and small molecules) can be covalently bound to GNP-COOH via a very straightforward
86 conjugation chemistry based on carbodiimide coupling.

87
88

89 **Materials and methods**

90 *Chemicals*

91 Polyclonal antibodies towards chicken immunoglobulins (Ab_{v,s}Clg) developed in rabbit and towards
92 mouse immunoglobulins (Ab_{v,s}Mlg) developed in goat, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide
93 hydrochloride (EDAC), bovine serum albumin fraction V (BSA), cadmium oxide (CdO, 99,99%), selenium
94 powder (Se, 99,99%), sulfur powder (S, 99%), zinc acetate (Zn(OAc)₂, 99,99%), oleic acid (OA, 90%), 1-
95 octadecene (ODE, 90%), oleylamine (OLA, 70%), octadecylamine (ODA, 90%), poly(maleic anhydride-alt-1-
96 octadecene) (PMAO, M ~ 30 000-50 000), rhodamine 6G, 4-aminophenylacetic acid, and HCl sodium nitrite
97 were purchased from Sigma-Aldrich (MO, USA). Mouse and chicken immunoglobulins were the fraction
98 obtained from the respective animal serum through sulfate ammonium precipitation. Jeffamine M1000
99 (1000 g/mol) was kindly provided by Huntsman (Belgium). Black well microplates and all other chemicals
100 were obtained from VWR International (PA, USA). Phosphate buffer saline (PBS, phosphate20mM, pH 7.4)
101 was used as the diluent for the homogenous assays.

102

103 *Graphene nanopowder functionalization*

104 Graphene nanopowder (GNP, Graphene Supermarket, 3 nm flakes, Grade AO1) was modified with
105 carboxylic groups using diazonium chemistry [18-19]. To prepare this sample, from now on called GNP-

106 COOH, the GNP powder was added to a solution containing 0.05 M 4-aminophenylacetic acid, 0.5 M HCl
107 and 0.05 M sodium nitrite. The reaction was carried out for ten minutes; then GNP-COOH was filtered
108 (Whatman cellulose acetate filter with 3.0 μm pores) and washed several times with deionized (DI) water
109 and isopropyl alcohol, and let dry overnight at 323 K. A complete characterization of the synthesized
110 material is reported elsewhere [17]).

111

112 *QD synthesis*

113 Hydrophilic core/shell/shell CdSe/CdS/ZnS QDs with emission at 636 nm (QDI) and at 607 nm (QDII)
114 and photoluminescence quantum yield $\sim 30\%$ were synthesized. First CdSe QDs with two different
115 diameters (4.4 nm and 3.7 nm) were prepared by a hot-injection method described in [24-26]. The
116 diameters of the QDs were determined on basis of the calibration curve from [27]. Then CdSe QDs were
117 covered with 3 layers of CdS and 2 layers of ZnS by SILAR (Successive Ionic Layer Adsorption and Reaction)
118 method [28, 29]. The solutions of precursors for shell growth were prepared as follows. The Cd precursor
119 solution (0.05 M) was prepared by combining CdO, oleic acid (molar ratio CdO:OA was 1:8) with ODE inside
120 a three-neck flask and heating at 210°C under argon atmosphere until the solution turned clear. The S
121 precursor solution (0.05 M) was made by dissolving S in ODE at 160 °C under argon. The Zn precursor
122 solution (0.05 M) was prepared in a glovebox by dissolving zinc acetate in a mixture of oleylamine and
123 octadecene ($v(\text{Zn})/v(\text{OLA})=1:8$) at 120 °C. For a typical synthesis 20 ml of ODE and 4.5 g of ODA were loaded
124 into a 100 ml reaction flask and heated at 100 °C under argon for 1 hour. About 3×10^{-4} mmol of CdSe core
125 nanocrystals in hexane was added to the flask and the mixture was flushed with argon for 1 hour to remove
126 the hexane. Then the flask was heated up to 225 °C. The S and Cd (or Zn) precursor solutions (0.05 M) were
127 added consecutively via syringe to the reaction flask containing the CdSe cores, waiting 10 min after each
128 injection. This was followed by alternating addition of Cd or Zn precursors and S precursor, respectively. We
129 determined the amount of the injection solution by method [27] based on the calculation of the number of
130 surface atoms. Upon completion of the synthesis, the reaction was cooled down to ~ 70 °C and small
131 amount of chloroform was added to prevent solidification of amine. Then the clear solution was transferred
132 to a centrifugation tube and acetone was added until opalescence appeared. The mixture was centrifuged
133 for 7 min at 2700 g. Afterwards the precipitate was dissolved in toluene and stored in a refrigerator under
134 Ar.

135 To transfer QDs to aqueous solution, we prepared the amphiphilic polymer (poly(maleic anhydride-
136 alt-1-octadecene) – Jeffamine M1000, PMAO-PEG): 2 g of Jeffamine M1000 was dissolved in 15 ml
137 chloroform and was added drop by drop to a flask containing 1 g of PMAO. The powder immediately
138 dissolved and the solution was refluxed at 60°C during 1 hour under nitrogen and then was left under
139 stirring overnight. Purified QDs and the amphiphilic polymer were mixed in chloroform and stirred
140 overnight at room temperature (mass ratio of QD:PMAO was $\sim 1:7$). An equal volume of KOH solution (0.05

141 M) was added to the QDs-polymer chloroform solution. Afterwards the chloroform was slowly evaporated
142 by a Bunsen's water-air-jet pump and a clear fluorescent solution was obtained. To remove the polymer
143 excess, chloroform was added drop by drop to QDs aqueous solution ($V(\text{CHCl}_3):V(\text{QDs solution})=1:1$). As a
144 result, a white precipitate at the chloroform-water interface was formed. The sample was centrifuged at
145 2700 g and the aqueous phase was taken away. The procedure was repeated 3-4 times until the next
146 chloroform addition did not provoke the formation of any visible white precipitate. After that the solution
147 was placed under reduced pressure (with water-jet pump) to remove chloroform residues. Excess of
148 Jeffamine M1000 was removed by three rounds of ultrafiltration with Amicon centrifuge filters (100kD
149 MWCO).

150 For the estimation of quantum yield (QY), the spectrally integrated emission of quantum dot
151 solutions were compared to the emission of dyes with known quantum yield (6 G: QY=95% at λ (exc.)=490
152 nm) as described in [30].

153

154 *Preparation of QD-antibody conjugates*

155 We prepared QDI-Ab_{vs}MIg and QDII-Ab_{vs}CIg as follows. 0.1 ml of QDs diluted in 1 ml of PBS were pre-
156 activated with 0.05 ml of a freshly prepared aqueous solution of EDAC (10 mg/ml) for 20 min at room
157 temperature. Then, we added 0.5 mg of Ab_{vs}MIg or Ab_{vs}CIg to the pre-activated QDI and QDII, respectively,
158 and incubated for 30 min at room temperature. A second aliquot of EDAC was added and the tubes were
159 transferred to 4°C for a further overnight incubation. To ensure the complete covering of QD surface with
160 proteins, we used 1 mg of BSA (0.1 ml of a 10 mg/ml PBS solution) to end-cap QD-antibody conjugates and
161 these were stored at 4°C until use. QD-antibody conjugates were characterized by recording emission
162 spectra on a CARY Eclipse fluorescence spectrophotometer (Agilent Technologies, CA, USA) before and
163 after conjugation. QD-labeled antibodies were separated from unbound antibodies by ultracentrifugation
164 at 16000 x g for 30 min.

165

166 *Preparation of GNP-protein conjugates*

167 GNP-COOH was reacted with both mouse and chicken immunoglobulins to prepare the GNP-COOH-
168 MIg-CIg conjugate. Briefly, 1 mg of GNP-COOH was dispersed in 0.4 ml of PBS by sonicating for 15 min, and
169 then activated by adding EDAC as described above. 0.5 mg of each immunoglobulin was then added and
170 incubated for 30 min at room temperature. A second EDAC aliquot was then added, the mixture was
171 incubated overnight at 4°C, and 1 mg of BSA was added to end-cap the GNP-MIg-CIg conjugate. The GNP
172 end product was recovered by centrifugation at 3000 x g for 5 min. The pellet was washed twice with PBS
173 and finally recovered in 1 ml of PBS. The suspension was sonicated for 15 min, diluted 1:100 with PBS and
174 stored at 4°C until use. We estimated the rate of immunoglobulins (either MIg or CIg) conjugated to GNP-

175 COOH by measuring immunoglobulins remaining in the supernatant, which proved to be undetectable.
176 Therefore, immunoglobulins were completely bound to GNP-COOH.

177
178 *Inhibition of QD fluorescence due to interaction with GNP-COOH-MIg-Clg*

179 The interaction between GNP-COOH-MIg-Clg and QD-antibody conjugates was evaluated by
180 recording the fluorescence emission on a FLx800 fluorescence microplate reader (BioTek, VT, USA). The
181 excitation and emission wavelengths were set at 360 (± 40) nm and 460 (± 40) nm, respectively.
182 Checkerboard titrations were adopted to establish amounts of QD-Ab_{vs}MIg or QD-Ab_{vs}Clg and GNP-COOH-
183 MIg-Clg. 10, 20, and 40 μ l of QDI-Ab_{vs}MIg or QDII-Ab_{vs}Clg were mixed with 10 μ l of the GNP-COOH-MIg-Clg
184 conjugate previously diluted at 1:10, 1:100, and 1:1000. PBS was added to reach an overall volume of 200
185 μ l. The fluorescence quenching due to the interaction between GNP-COOH-MIg-Clg and QD-_{vs}MIg or
186 QD-_{vs}Clg was observed between 10' and 90'. The emission of QD-Ab_{vs}MIg or QD-Ab_{vs}Clg alone and mixed
187 with GNP-COOH were measured too, to check for non-specific binding and other non-specific causes of
188 fluorescence decay.

189
190 *Homogeneous assays based on QD fluorescence quenching by GNP*

191 To check the efficacy of the competitive assay, we first tested a model including only a single analyte.
192 We put in contact different amounts of either antigen (MIg or Clg) with fixed amounts of the QD
193 functionalized with the corresponding antibody in the presence of the GNP-COOH-MIg-Clg. More
194 specifically, 40 μ l of QDI-Ab_{vs}MIg or 20 μ l QDII-Ab_{vs}Clg were mixed in the wells of a microplate reader with
195 10 μ l of GNP-COOH-MIg-Clg (diluted 1:100) and with 100 μ l of either MIg or Clg (0-1.5-7.5-30-100-500
196 mg/l), respectively. PBS was added to reach an overall volume of 200 μ l, and the mixture was incubated for
197 30' at room temperature. The emitted fluorescence was then recorded as indicated above.

198 To assess the possibility of establish a multi-analyte assay, based on the unique optical property of
199 QDs that can be excited at the same wavelength and emit light at different wavelengths, experiments were
200 conducted by employing the same protocol, except that all volumes were doubled and QDI-Ab_{vs}MIg, QDII-
201 Ab_{vs}Clg, GNP-COOH-MIg-Clg and both antigens were all mixed together. Fluorescence was recorded on the
202 CARY Eclipse fluorescence spectrophotometer, with excitation wavelength of 350 nm, and emission
203 recorded between 550 and 680 nm.

204 Standard curves were obtained by plotting fluorescence emitted at 594 nm against Clg concentration
205 and fluorescence emitted at 634 nm against MIg concentration, based on the emission maxima of QDI and
206 QDII, respectively. Curves obtained by subtracting the signal of the blank (no target protein in the sample)
207 to the signals of solutions containing different protein amounts were fitted by a 4-parameter logistic
208 equation.

209

210 **Results and Discussion**

211 The multianalyte “turn on” fluoroimmunoassay that we propose is based on the competitive binding
212 of free antigens and QD-labelled antigens on GNP on which we have immobilized Clg and MIg. The binding
213 between Clg and MIg on GNP is mediated by COOH groups covalently bound to GNP using diazonium
214 chemistry (Figure 2). Briefly, carboxylate groups are added to GNP starting from 4-aminophenylacetic acid
215 as precursor, as previously described by Sordello et al [17] (Figure 2a). The carboxylate groups then react
216 with amino groups present in Clg and MIg with mediation of EDAC, and a covalent amide bond is formed
217 between Clg and MIg and GNP at the end of the process (Figure 2b).

218 Successful conjugation between QDs and antibodies is shown by the changes in QD emission spectra
219 (Figure 3): when excited at 230 nm, unmodified QDI and QDII show emission maxima at 636 nm and 607
220 nm, respectively (Figures 3 a and b, continuous line spectra); after conjugation with antibodies (Figure 3 a
221 and b, dashed lines), a further emission band shows up at about 330 nm for both QDs, which is attributable
222 to the fluorescence of the covalently bound antibodies [31]. Nevertheless, the second harmonic of
223 fluorescence due to antibodies interfered with QD emission. To avoid this interference, we set the
224 excitation wavelength at 350 nm (Figures 3 c and d). In these conditions, only the contribute to
225 fluorescence due to QDs was observed and emission maxima of QD conjugated to antibodies (dashed lines)
226 were observed at 634 and 594 nm for QDI and QDII, respectively . A further less intense emission was also
227 observed at about 440 nm for both QDs when conjugate to antibodies and excited at 350 nm.

228 The conjugation with antibodies should yield QDs able to selectively bind the corresponding antigen
229 present on GNP, thus bringing the QDs and the GNP close enough to allow for energy transfer and
230 quenching of QD fluorescence. We studied the interaction between QD-Ab_{vs}MIg and QD-Ab_{vs}Clg and GNP-
231 COOH-MIg-Clg by measuring QD fluorescence as a function of the QD/GNP ratio and contact time (Figure
232 4). As control samples, we use bare QDs in interaction with GNP-COOH-MIg-Clg, as well as QD-Ab_{vs}MIg and
233 QD-Ab_{vs}Clg alone. All the results are summarized in Table 1, and Figure 4 shows those obtained for QD-
234 Ab_{vs}MIg as an example. When QDs and functionalized GNP are mixed, the fluorescence decreases almost
235 linearly as a function of time. The combination of conjugated QDs and GNP-COOH-MIg-Clg yielded a more
236 evident fluorescence decrease, which depended on GNP-COOH-MIg-Clg amount (namely on QD-Ab/ GNP-
237 COOH-MIg-Clg ratio): GNP-COOH-MIg-Clg diluted 1:100 was the most effective in inducing fluorescence
238 quenching, followed by the 1:10 dilution. The 1:1000 dilution showed a slower decay in QD emission,
239 however significantly different from that observed for the control systems. We expected a lower efficiency
240 in fluorescence quenching when decreasing the amount of GNP-COOH-MIg-Clg, however, the 1:10 dilution
241 showed less quenching than the 1:100 dilution. We hypothesize that higher GNP-COOH-MIg-Clg
242 concentrations lead to less stable suspensions or to the establishment of interactions within GNP-COOH-
243 MIg-Clg sheets, which reduce their availability for binding to conjugated QDs.

244 Control samples show a much lower decrease in fluorescence when unconjugated QDs are mixed
245 with GNP-COOH-MIg-Clg (Figure 4 a-c, grey symbols), and an almost constant fluorescence emission for
246 both conjugated and unconjugated QDs in the absence of GNP-COOH-MIg-Clg, except for a minor decrease
247 in the first 30 minutes (Figure 4d). This indicates that some non-specific interactions between QDs and GNP
248 may occur, which cause a delayed reduction of QD emission. Nevertheless, the faster fluorescence
249 reduction observed for the QD-Ab_{vs}MIg and QD-Ab_{vs}Clg when mixed with GNP-COOH-MIg-Clg systems
250 proves the establishment of the antibody-antigen binding.

251

252 *Homogeneous immunoassay for a single analyte*

253 After showing that the binding of QDs conjugated to antibodies to functionalized GNP does indeed
254 quench QD fluorescence, we can proceed to test the homogeneous competitive immunoassay described
255 above (Figure 1). The closeness of QDs to GPN, due to the binding between antibodies linked to QDs and
256 their antigens linked to GPN sheets, determined a strong quenching of the QD fluorescence, therefore a
257 homogenous competitive immunoassay could be designed, based on the inhibition of QD/GPN binding in
258 the presence of the antigen in the same solution (Figure 1). The addition of increasing amounts of antigens
259 to the QD-Ab_{vs}MIg and QD-Ab_{vs}Clg /GNP-COOH-MIg-Clg mixture should cause a parallel increase in QD
260 fluorescence, since the QDs conjugated to antibodies should bind to the free antigen and be displaced from
261 GNP-COOH-MIg-Clg.

262 We defined the optimal conditions for the homogenous competitive immunoassay using a standard
263 checkerboard assay, in which we selected the appropriate competitor concentrations (i.e. the amount of
264 functionalized GNP) and antibody dilutions (i.e. the amount of QD conjugated to antibodies) based on the
265 lowest IC₅₀. We ran these tests as microplate assays, separately on QDI/MIg and QDII/Clg systems. We
266 optimized the incubation time by comparing dose-response curves obtained after 30, 60 or 90 min of
267 reaction. We also checked the possibility of cross-reaction within the two systems, i.e. the possibility that
268 QDI-Ab_{vs}MIg could bind to Clg and *vice-versa*. The results are summarized in Figure 5, which shows typical
269 inhibition curves for the two systems obtained by plotting fluorescence against concentrations of the target
270 antigens and non-specific antigens at different incubation times. Figure 5a shows inhibition curves obtained
271 by mixing QDI-Ab_{vs}MIg and the functionalized GNP with increasing amounts of MIg (solid symbols), which
272 represented the target antigen, and with Clg (open symbols) to assess cross-reactivity, while Figure 5b
273 shows inhibition curves obtained for QDII-Ab_{vs}Clg, in the presence of its target analyte (Clg, open symbols)
274 and with the potentially cross-reagent MIg (solid symbols). Both systems proved to respond to the
275 presence and quantity of their respective antigen, as expected, whereas cross-reactivity between the two
276 targets resulted negligible in all cases. Based on these results, we selected 30 min incubation as a
277 satisfactory compromise between the lowest IC₅₀ and data precision.

278

279 *Multi-analyte homogenous assay*

280 After showing that our competitive assay can measure the presence of one analyte in solution, we
281 tested the possibility of using it to determine two analytes at the same time by measuring fluorescence at
282 two wavelengths. We mixed together the two QDs conjugated to antibodies (QDI-Ab_{vs}MIg , QDII-Ab_{vs}CIg),
283 GNP-COOH-MIg-CIg and the two target antigens (MIg and CIg) and incubated the mixture for 30 min at
284 room temperature. Then we measured the fluorescence emitted between 550 and 680 nm, using an
285 excitation wavelength of 350 nm. The increment of each antigen in the mixture induced the increase of the
286 emitted fluorescence at the expected wavelength (Figure 6). A partial overlap of emission was observed,
287 due to the fact that QDI and QDII have close emission maxima (594 and 634 nm). Nevertheless, calibration
288 curves for the two targets could be obtained by plotting fluorescence values (arbitrary units) measured at
289 634 and 594 nm against MIg and CIg concentrations, respectively (Figure 7). The developed assay allows
290 measuring both antigens in a wide concentration interval (two-three orders of magnitude) and showed
291 estimated limits of detection in the nanomolar range.

292

293 **Conclusions**

294 We developed a competitive homogenous immunoassay, based on labelling antibodies with
295 quantum dots and exploiting the quenching of QD fluorescence by resonance energy transfer to graphene.
296 We functionalized graphene with carboxylic groups (GNP-COOH), which allowed for covalent linking of
297 antigens to graphene sheets. The establishment of the antibody-antigen interaction brings QD and
298 graphene close enough to permit the energy transfer and, thus, the quenching of QD fluorescence. The
299 competition between free and graphene-linked antigens causes the displacement of QD-labelled antibodies
300 from graphene. The presence of free antigens thus restores QD fluorescence, acting as a “turn on” input.
301 The emitted light is linearly related to the free antigen amount. This method is universal, i.e. it potentially
302 can work with analytes of any molecular weight provided that they can be covalently linked to GNP-COOH
303 through its carboxylic functional groups, differently from previously reported methods [2,5]. The detection
304 itself is fast enough as the assay is fully homogeneous, whereas heterogeneous immunoassays involve
305 multiple steps of incubation. The stability of the fluorescence of conjugated QDs in the absence of related
306 antigens allows using their fluorescence intensity as an internal calibration standard, thus simplifying result
307 analysis. Finally, the proposed strategy is applicable in a multi-analyte configuration.

308

309 **Acknowledgements**

310 We acknowledge support from a Marie Curie International Research Staff Exchange Scheme Fellowship
311 (PHOTOMAT, proposal n° 318899) within the 7th European Community Framework Programme, and from
312 the Canada Research Chair foundation.

313

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385

386 **TABLES**

387 Table 1. Linear fit of QD fluorescence decay due to the interaction between QD conjugated to
 388 antibodies (QDI-Ab_{vs}Mlg and QDII-Ab_{vs}Clg) and GNP functionalized with the corresponding antigen as a
 389 function of time of contact for various GNP-COOH-Mlg-Clg amounts. As a control quenching of non-
 390 conjugated QDs (QDI and QDII) was also evaluated.

391

	GNP-COOH-Mlg-Clg dilution		
	1:10	1:100	1:1000
QDI	$y=-1.23x+246,$ $r^2=0.968$	$y=-1.02x+287,$ $r^2=0.926$	$y=-1.18x+170,$ $r^2=0.962$
QDI-Ab _{vs} Mlg	$y=-1.74x+246,$ $r^2=0.992$	$y=-2.03x+294,$ $r^2=0.997$	$y=-1.51x+229,$ $r^2=0.995$
QDII	$y=-1.39x+297,$ $r^2=0.983$	$y=-1.13x+342,$ $r^2=0.967$	$y=-1.21x+206,$ $r^2=0.974$
QDII-Ab _{vs} Clg	$y=-1.55x+277,$ $r^2=0.983$	$y=-2.07x+190,$ $r^2=0.998$	$y=-1.53x+194,$ $r^2=0.993$

392

393

394 **FIGURES**

395

396 **Figure 1.** Schematic of the multi-analyte homogeneous immunoassay based on fluorescence
397 quenching of two QDs conjugated with antibodies by means of GNP-COOH covalently linked to
398 corresponding antigens. Mouse Immunoglobulins (MIg) and Chicken Immunoglobulins (CIg) are coupled to
399 GNP-COOH and interact with 2 QDs labeled with antibodies developed towards MIg and CIg respectively
400 (1). When light is shined on this configuration, no fluorescence is observed because the QD fluorescence is
401 quenched by GNP (2). The addition of antigens, one at a time (3-4) or mixed together (5), restores
402 fluorescence of the respective QD.

403

404 **Figure 2.** GNP functionalization to yield GNP-COOH (a) and its covalent modification to prepare GNP-
405 COOH-MIg-CIg (b)

406

407 **Figure 3.** Emission spectra of QD before (continuous lines) and after conjugation (dashed lines) with
408 antibodies: excitation was set at 230 nm (a and b) or 350 nm (c and d)

409

410 **Figure 4.** Quenching of QDII fluorescence due to the interaction between unconjugated QD (grey
411 symbols, ■) or QD conjugated to antibodies (black symbols, ◆) and GNP functionalized with the
412 corresponding antigen as a function of time of contact and for various functionalized GNP amounts (a-c). As
413 a further control, QD and QD-Ab_{v5}CIg were also incubated in the absence of functionalized GNP (d).

414

415 **Figure 5.** Inhibition of binding of conjugated QDs to GNP-COOH-MIg-CIg by MIg (solid symbols) or CIg
416 (open symbols) added to the solution after increasing incubation times: 30' (●), 60' (■), and 90' (▼). QDI-
417 Ab_{v5}MIg (a) and QDII-Ab_{v5}CIg (b) were separately incubated with increasing amounts of each of the two
418 antigens and the emitted fluorescence was measured by means of a fluorescence microplate reader
419 (excitation: 360±40 nm, emission: 460±40 nm).

420

421 **Figure 6.** Emission spectra recorded by means of a fluorescence spectrophotometer (excitation 350
422 nm) for the multi-analyte system. QDI-Ab_{v5}MIg and QDII-Ab_{v5}CIg were mixed with the GNP-COOH-MIg-CIg
423 and target analytes at two concentration levels: 0 (solid line) and 500 mg l⁻¹ (dashed line).

424

425 **Figure 7.** Typical calibration curves obtained in the multi-analyte assay configuration by plotting
426 fluorescence emitted at 594 nm (●) and 634 nm (■) against CIg and MIg concentrations, respectively.
427 Measured fluorescence (arbitrary units) in the absence of any targets (FU₀) was subtracted to the
428 fluorescence measured at each individual target concentration. Inset shows actual measured fluorescence.

429 FIGURES

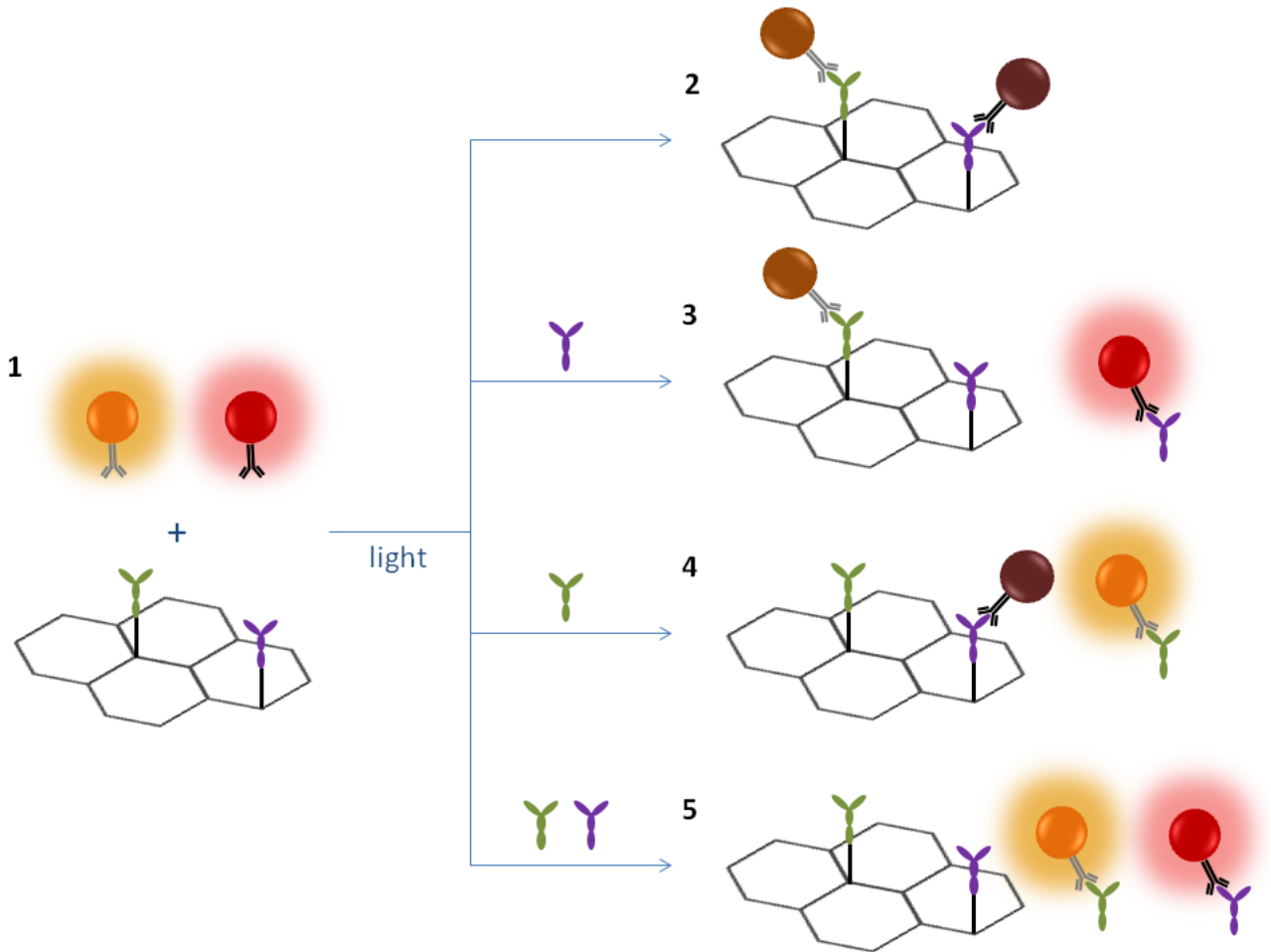
430 Figure 1

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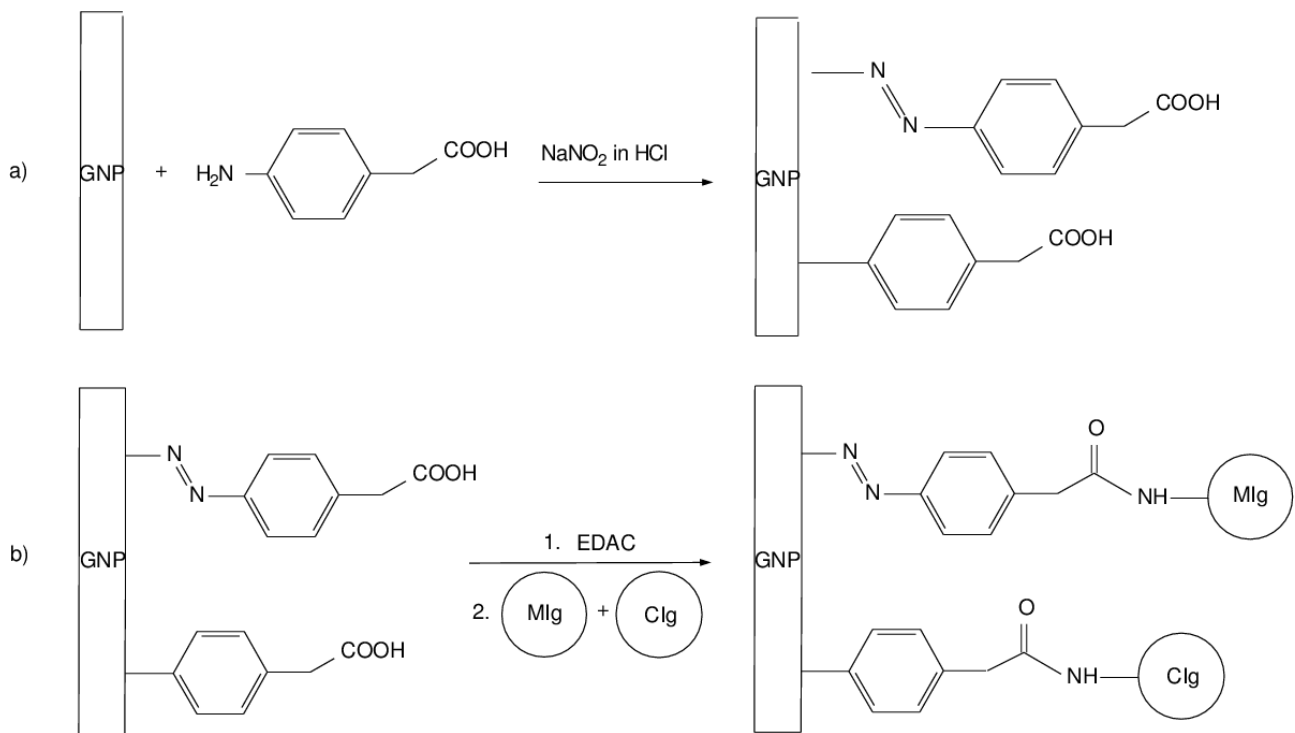
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437 Figure 2
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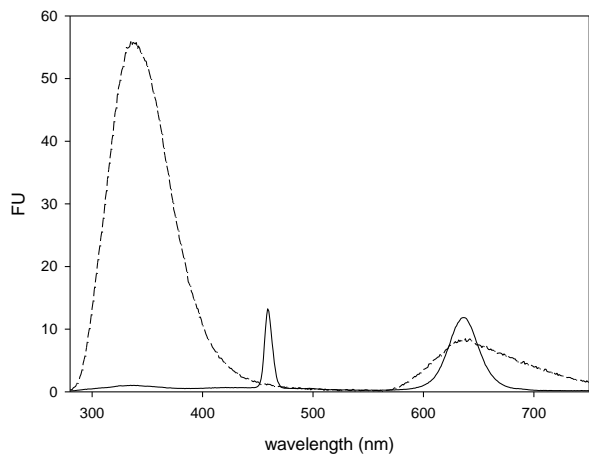
442 Figure 3

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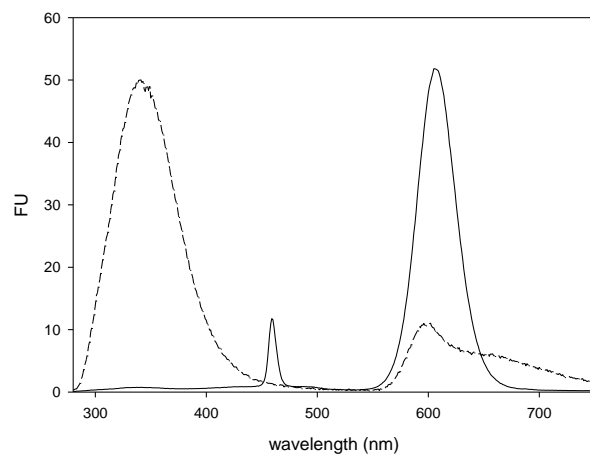
445 a

QDI, ex 230 nm



b

QDII, ex 230 nm



448

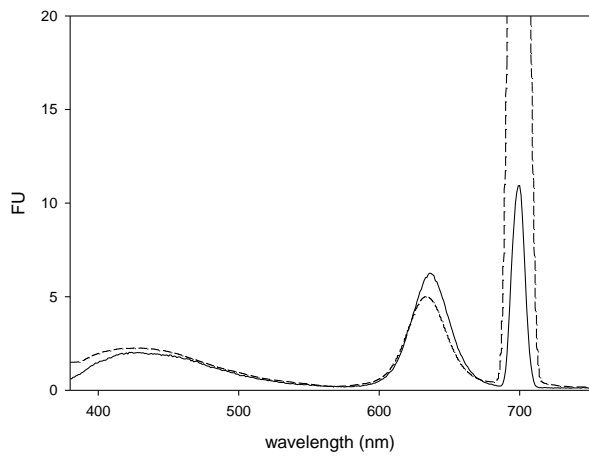
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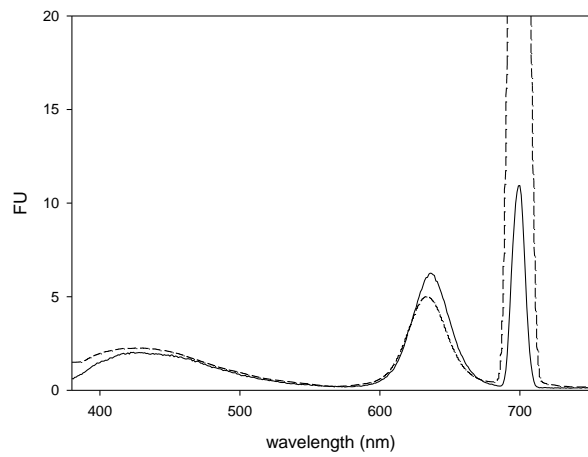
452 c

QDI, ex 350 nm



d

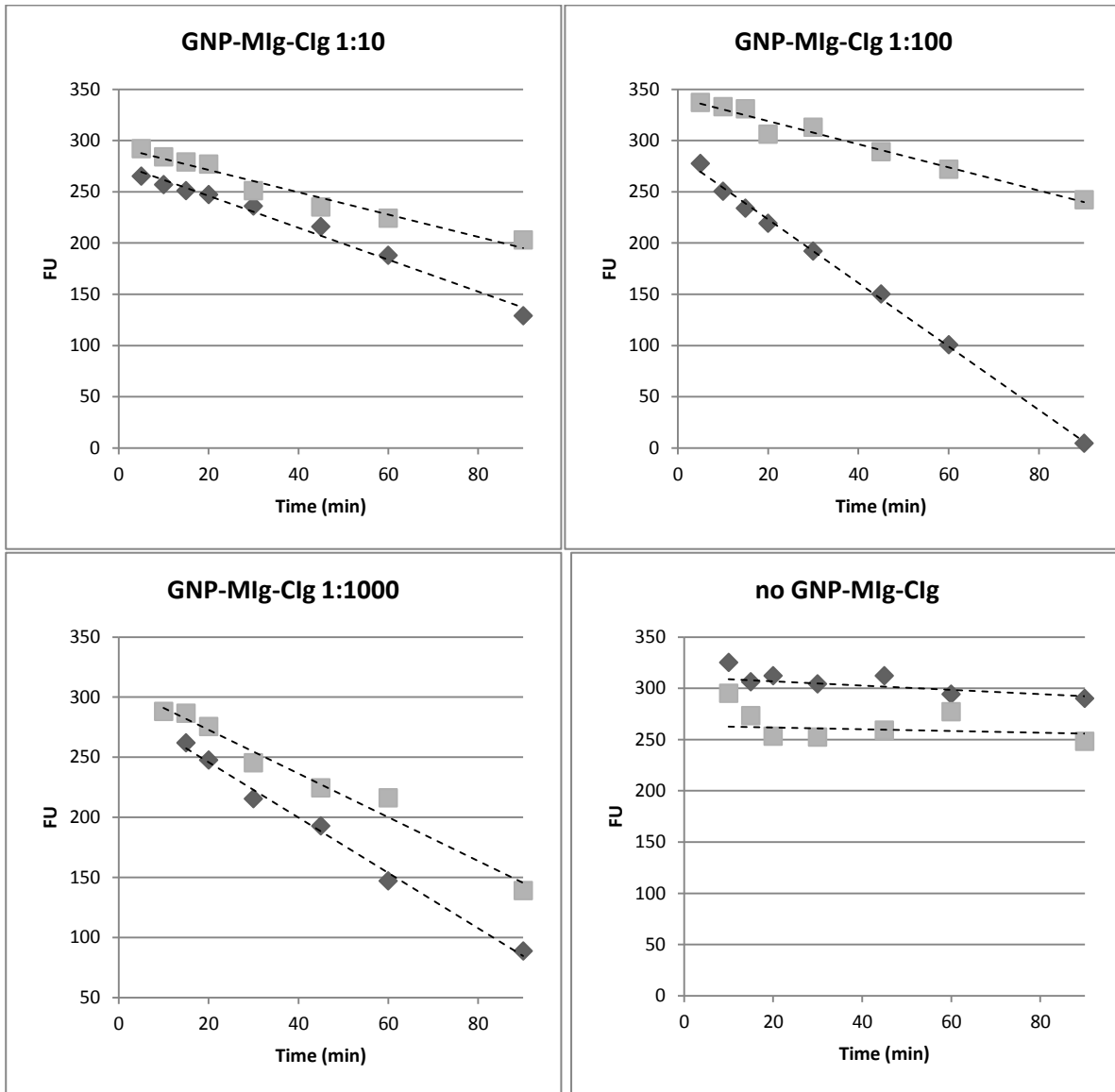
QDII, ex 350 nm



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456 Figure 4
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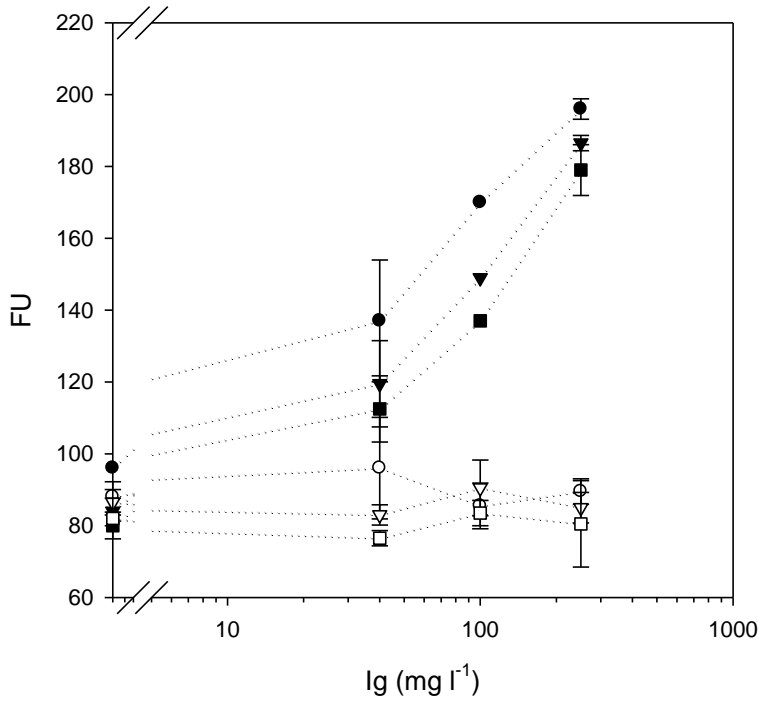
462 Figure 5

463

464 a

465

QDI-Ab-vs-MIg



466

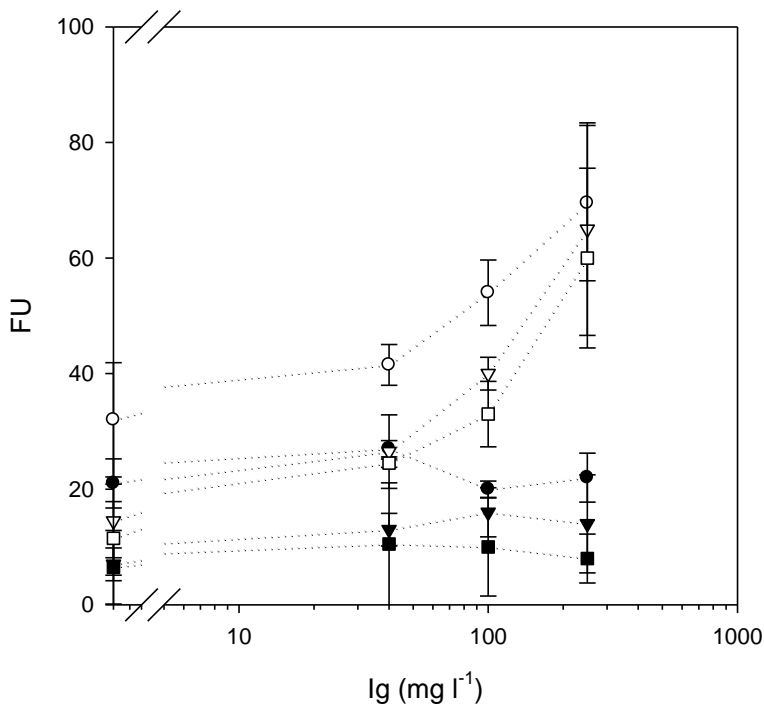
467

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469 b

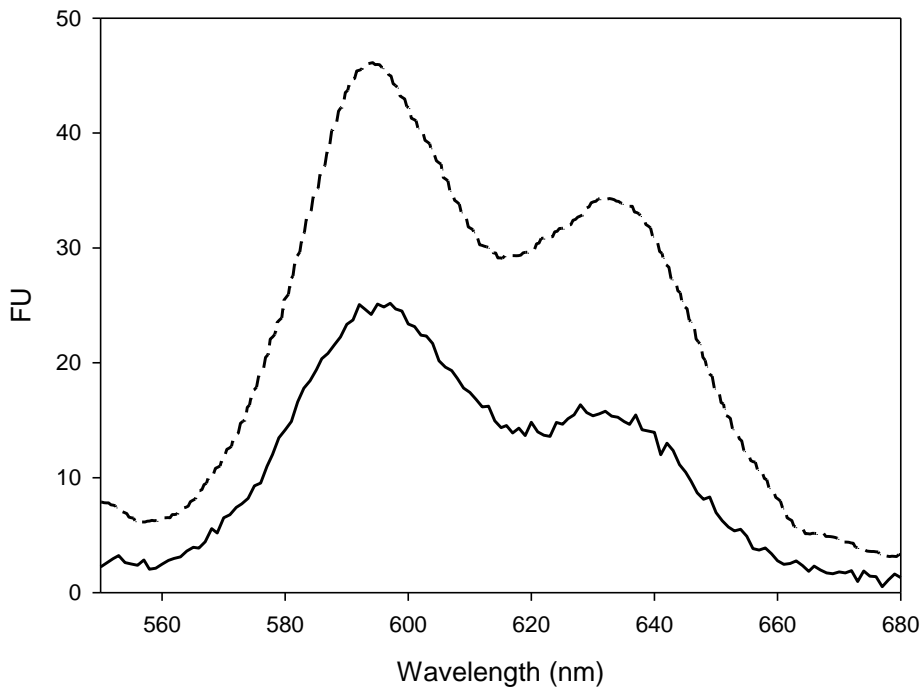
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QDII-Ab-vs-CIg



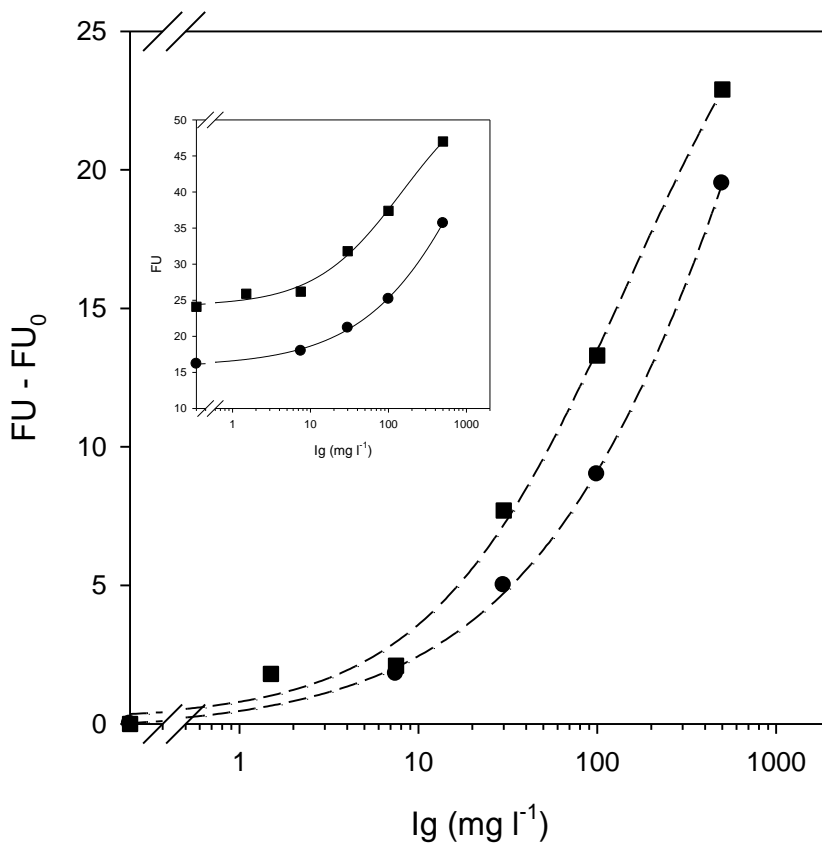
471

472 Figure 6



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475 Figure 7
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