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Quenching of Fluorescence Caused by Graphene Oxide as an Immunosensing Platform in a Microwell Plate Format †

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† Presented at the 1st International Electronic Conference on Biosensors, 2–17 November 2020; Available online: https://iecb2020.sciforum.net/.

Received: date; Accepted: date; Published: date

Abstract: The immunoassays are nowadays an important tool for diagnostics, drug development, and environmental monitoring. However, most immunoassays involve procedures that require many elements for their development. We introduce a novel biosensing platform based on fluorescence quenching caused by graphene oxide (GO) for the detection of Human-IgG and Prostate Specific Antigen (PSA). It was used a single antibody (conjugated with the FITC fluorophore for H-IgG detection and conjugated with Qdots for PSA detection) for capture and detection processes and avoiding washing steps. The simple biosensing platform consists of the covering of a 96 wells microplate (with polystyrene bottom) with GO. The graphene oxide adhesion is possible by way of electrostatic interactions between the plate surface modified with amino groups (positively charged) and the graphene oxide (negatively charged). This proposal showed excellent response for the detection of Human-IgG, with acceptable precision (from 0.27% to 5%). The limit of detection reached for H-IgG was 3.35 ng mL^{-1}. In the same manner, for PSA detection, the limit of detection reached was 0.02 ng mL^{-1} and the precision range was from 0.7% to 15.2%. Furthermore, this biosensing platform was demonstrated to operate with real samples of human urine doped with different concentrations of prostate-specific antigen.

Keywords: FRET; fluorescence; immunoassays; graphene; single-step biosensing platform

1. Introduction

Nowadays immunoassays are used to detect chemical or biological species; therefore, they are an essential tool in a wide range of applications such as drug development, clinical diagnostics, environmental monitoring, or food quality control [1]. However, conventional immunoassays, such as ELISA (Enzyme-Linked ImmunoSorbent Assay), require several procedures such as blocking, separations, and washing steps. Thus, it takes at least 6 h to get the respective results. Besides, it involves two antibodies and a sensing surface attaching and labeling the biochemical target (analyte). Fluorescence Resonance Energy Transfer (FRET) is a very useful phenomenon to improve immunosensing sensitivity and avoid cumbersome procedures due to its simplicity. Graphene and its derivatives have been used as acceptors in FRET due to their wide absorption spectra, which make them outstanding quenchers of fluorescence [2]. With this in mind, we developed a novel and single-step biosensing platform based on fluorescence quenching caused by graphene oxide, which was

used for the detection of two analytes: H-IgG (which is a type and also the most common antibody found in human blood circulation) and PSA (prostate-specific antigen). A single antibody conjugated with a fluorophore (FITC for H-IgG detection and quantum dots for PSA detection) is used in the capture and detection processes. When the analyte and the antibody (conjugated with the fluorophore) are added, a kinetic analysis is performed for 2 h with real-time interrogation of the respective fluorescence intensity, observing that the higher the analyte concentration, the less quenching of fluorescence of the immunosensing probe (antibody-fluorophore immunocomplex). This is due to the low affinity and the relatively long distance between GOμWs (microwells plate coated with Graphene Oxide) and immunosensing probe [3].

2. Materials and Methods

2.1. GO-Coated Microwell (GOμW) Plates

100 μL of GO per microwell was added in microwell plates and the plates were left overnight aiming at coating the microwell surface with GO, see Figure 1a. The microwell's surface of the plate is modified with amino groups (thus positively charged); following specification afforded by the supplier, and GO has a negative charge on aqueous suspension. So, the coating of GO onto every microwell's surface is achieved principally via electrostatic interactions. After that time, three washing steps are made on every microwell plates with intention of remove excess of GO that did not attach, Figure 1b. In this way, the GOμW-based biosensing platform is ready-to-use.

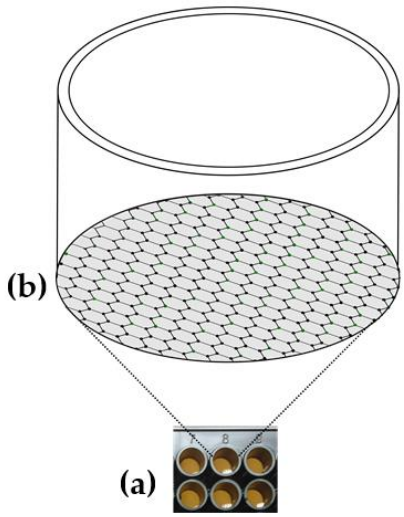

Figure 1. (**a**) microwell plates with GO in aqueous suspension (**b**) microwell covered with GO after washing steps.

2.2. GOμWs Based Immunoassay

Having biosensing platform ready, the detection is conducted by adding 50–100 μL of the photoluminescent biorecognition probe (antibody-fluorophore immunocomplex) and 50–100 μL of the analyte to be detected, in a GOμW. We use in every immunoassay seven concentrations of analyte (serial dilutions), a blank (no analyte), and three parallel experiments are made for every concentration to evaluate the precision of the measurement. The microplate reader records the intensity of fluorescence of every GOμW every 5 min during a kinetic analysis of 2 h. FITC conjugated with antibody is excited at 485 nm and has a maximum emission at 528 ± 10 nm, and Qdot conjugated antibody is excited at 365 nm and has a maximum emission at 665 ± 10 nm. For immunoassays using human urine, these samples are diluted using a 1/4 ratio before the analysis.

2.3. Conjugation of QDs with Anti-PSA

A concentration of 8 nM of Qdot Streptavidin conjugate is mixed with concentrations at μg mL^{-1} range of anti-PSA conjugated Biotin for 45 min under gently shaking. Both bioreagents are diluted in immunobuffer.

3. Results and Discussion

3.1. Characterization of Graphene Oxide-Coated Microwell Plates

With the intention of test the photoluminescence quenching capabilities of GOμWs produced under the experimental section, we conducted a kinetic analysis of 5 h with readings every 5 min of photoluminescence intensity. Compared with bare microwells (μWs), GOμWs with different GO concentrations (800, 1600, 3200 μg mL^{-1}) are capable to quench the photoluminescence of FITC conjugated with antibodies at a concentration of 1 μg mL^{-1}, Figure 2a. Photoluminescence intensity is normalized regarding the initial intensity I_0 to observe the quenching level at a certain time concerning such initial value.

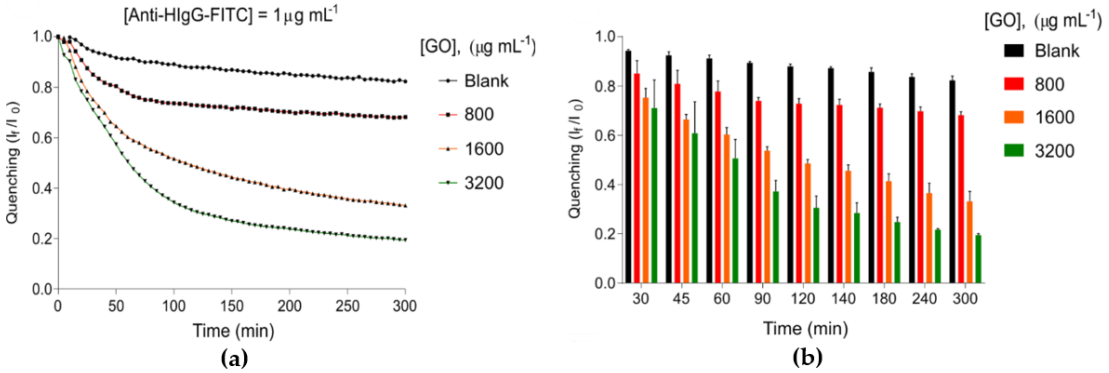

Figure 2. The photoluminescence quenching capabilities of GOμWs produced with different GO concentrations: (**a**,**b**) Experimental evidence of photoluminescent probes (FITC-labeled antibody) quenching monitored throughout 300 min. Adapted with permission from [3], Copyright 2020, Elsevier.

As shown in Figure 2b, the higher the GO concentration, the less quenching of photoluminescence. On the other hand, for bare μWs, the weak quenching of fluorescence (around 20% less respect to the initial value) is owing to the photobleaching phenomenon.

3.2. The Immunosensing Platform Targeting Human IgG

With the biosensing platform ready, we carried on an immunoassay to detect and to explore the interaction of several concentrations of Human IgG (H-IgG) with FITC-conjugated anti-Human IgG (Anti-H-IgG) when are mixed within GOμWs, following the experimental parameters. Figure 3a shows that for all concentrations of H-IgG there is a difference in levels of photoluminescence quenching concerning the blank throughout the kinetic analysis.

From 60 min, it is evident that starts to have the biggest difference in fluorescence quenching levels between analyte concentrations, as to depict in Figure 3b. Table 1 shows some validation parameters for H-IgG detection.

Table 1. Parameters of validation for H-IgG detection.

Limit of Detection ng mL^{-1}	Optimal Concentration GO μg mL^{-1}	Optimal Concentration Anti-H-IgG μg mL^{-1}	Range of Coefficient of Variation (Precision)
3.35 ± 1.08	1600	0.125	0.27–5%

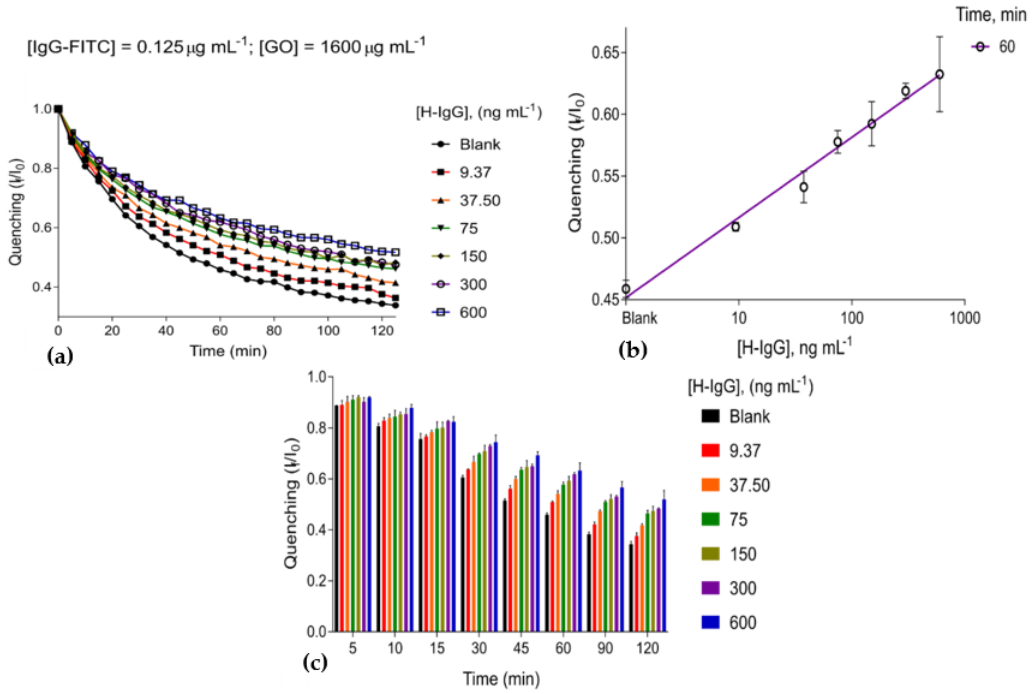

Figure 3. Analytical performance of the optimized immunosensing platform targeting H-IgG: (**a**) Real-time analysis of different concentrations of H-IgG and blank sample; (**b**) Calibration curve resulting at 60 min of the proposed immunoassay; (**c**) Behavior of the sensitivity of the resulting immunoassay across specific times. Adapted with permission from [3], Copyright 2020, Elsevier.

3.3. The Immunosensing Platform Targeting Prostate-Specific Antigen

We also designed a configuration of the immunosensing platform for the detection of Prostate Specific Antigen (PSA) to evince that this enjoys highly transformative capabilities. In this configuration, the photoluminescence probe is composed of Streptavidin-Qdots and biotinylated monoclonal antibody (anti-PSA). It is worth mentioning that a serum PSA level of up to 4 ng mL^{-1} is generally considered normal. Figure 4a depicts the kinetic analysis behavior of fluorescence intensity for PSA concentrations used. Some validations parameters for PSA detection are shown in Table 2.

Table 2. Parameters of validation for PSA detection.

Limit of Detection ng mL^{-1}	Optimal Concentration GO μg mL^{-1}	Optimal Concentration Qdot nM	Optimal Concentration Anti-PSA μg mL^{-1}	Range of Coefficient of Variation (Precision)
0.02	1400	0.05	0.02	0.7–15.2%

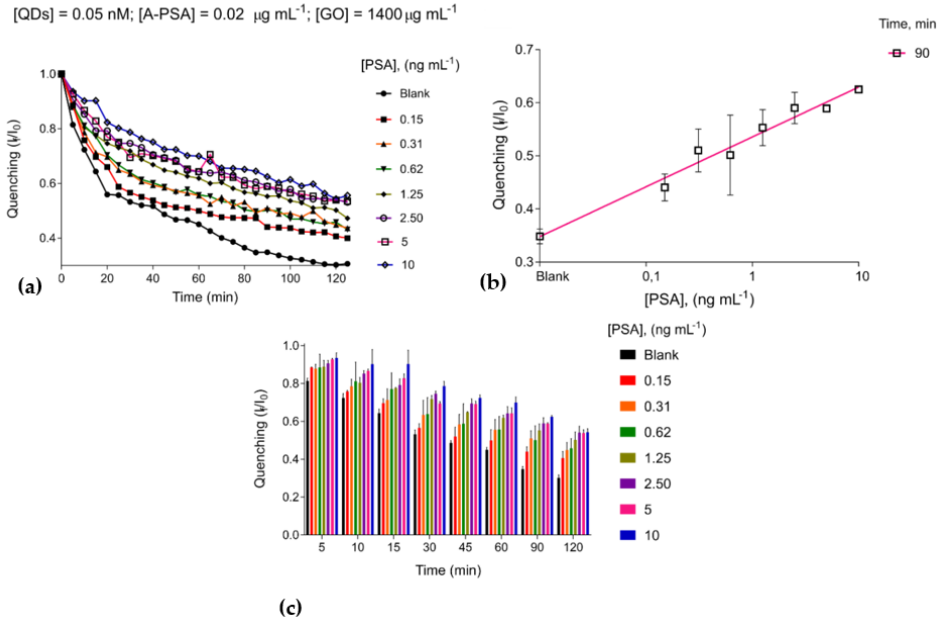

Figure 4. Analytical performance of the optimized immunosensing platform targeting PSA: (**a**) Real-time analysis of different concentrations of PSA and blank sample; (**b**) Calibration curve resulting at 90 min of the proposed immunoassay; (**c**) Behavior of the sensitivity of the resulting immunoassay across specific times. Adapted with permission from [3], Copyright 2020, Elsevier.

3.4. Proof of Concept of Immunosensing Platform

Finally, we tested if the biosensing platform proposed could detect urine samples doped with several concentrations of PSA (from 0 to 10 ng mL^{-1}), as to depict Figure 5a. According to Figure 5c, the best analytical performance regarding the limit of detection occurs before 20 min of the assay.

It is evident that the biosensing configuration is saturated from 2.5 ng mL^{-1}, see Figure 5b,c. This is due to a matrix effect undergone in the proposed immunoassay that harm antibody binding and assay performance. However, the analytical performance of the immunoassay quickly increases until 10 min and then decrease around 20 min. Some validations parameters for PSA (in human urine) detection are shown in Table 3.

Table 3. Parameters of validation for PSA detection in human urine.

Limit of Detection ng mL^{-1}	Optimal Concentration GO μg mL^{-1}	Optimal Concentration Qdot nM	Optimal Concentration Anti-PSA μg mL^{-1}	Range of Coefficient of Variation (Precision)
0.05	1400	0.05	0.02	0.2–9.3%

4. Conclusions

Taking advantage of the famous microwell plate format and the outstanding photoluminescence quencher represented by GO, we developed a breakthrough in immunosensing utilizing the studied graphene oxide-coated microwells. Given the proposed operational principle, as detailed in the introduction, the studied immunosensing platform enjoys a sensitivity comparable to that of ELISA and acceptable precision and accuracy. On the other hand, the biosensing platform proposed to operate not only with different analytes but also with organic dye-decorated antibodies and streptavidin-functionalized QDs conjugated to biotinylated antibodies. The principal advantages of the innovative real-time immunosensing platform are that operate with a single antibody and avoiding washing, blocking, and separation steps; thus, saving valuable reagents and time. The studied immunosensing platforms targeting H-IgG and PSA, respectively, reached their best analytical performance before 120 min of the assay.

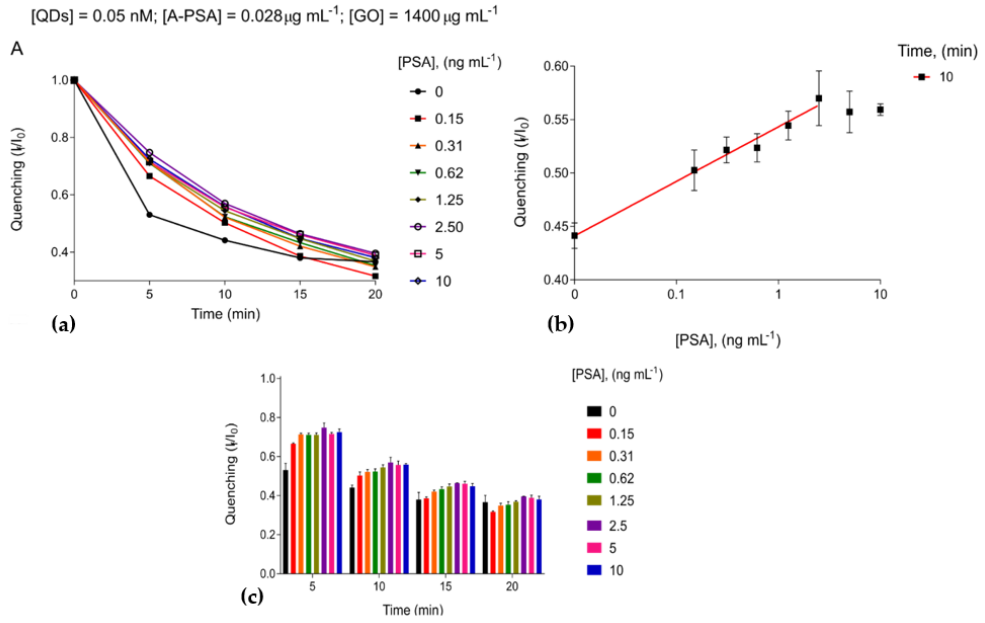

Figure 5. Analysis of human urine doped with different concentrations of PSA: (**a**) Real-time analysis of different concentrations of PSA in urine; (**b**) Calibration curve resulting at 10 min of the proposed immunoassay; (**c**) Behavior of the sensitivity of the resulting immunoassay across specific times. Adapted with permission from [3], Copyright 2020, Elsevier.

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