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Proceeding Paper

Smartphone-Adapted Multianalyte Biosensor Platform for Fluorescent Analysis of Human Biomarkers and Immunosuppressive Drugs Using PQQ- and NAD$^+$-Dependent Enzymes †

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Abstract: Here, we describe a multianalyte biosensor platform for the fluorescent analysis of different human state biomarkers (α-amylase, phenylalanine, glucose, lactate/pyruvate, alcohol) and some immunosuppressive drugs (cyclosporine A, tacrolimus, methotrexate, rapamycin) using chimeric PQQ- and natural NAD$^+$-dependent enzymes. The principle of the approach is based on the analysis of the brightness of photography of a sensor plate taken with a smartphone camera and processed using ImageJ software. The brightness of the image correlates with the fluorescence intensity of the sensor's spots which is produced by the enzymatic reduction of phenazine methosulfate or its derivative used as a fluorescence probe at UV 356 nm irradiation, where the amount of the reduced dye depends on the concentration of the target analyte (the enzymatic substrate) in the tested sample. The sensor plate is composed of simple and cheap components, and the procedure of its preparation and usage is easy and does not require any specific skills or expensive instrumentation. The proposed sensor platform is characterized by a high selectivity and storage stability depending on the selectivity and stability characteristics of the used enzyme in an immobilized state. The proposed sensor platform could be used for precision quantitative analysis of a single (or several) analytes or used for a simultaneous qualitative multianalyte assay of them using Boolean logic gates.

Keywords: smartphone-based biosensor; fluorescent analysis; PQQ- and NAD$^+$-dependent enzymes; human biomarkers; immunosuppressive drugs

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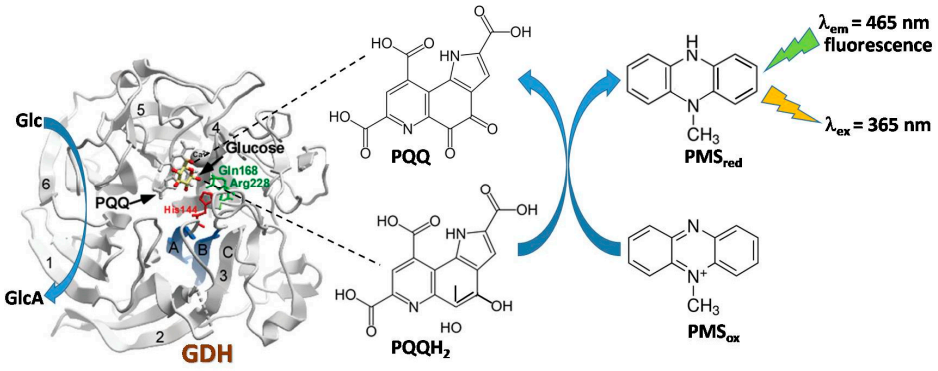
1. Introduction

The accurate analysis of trace amounts of some immunosuppressive drugs in patients and the main biomarkers indicating the human physiological state is a current requirement of modern clinical diagnostics and medicine. Most of the available techniques for such analysis require valuable equipment, complicated sample pretreatment, and time-consuming technical procedures processed by highly qualified personnel in clinical laboratories. From this point of view, the developments of portable, cheap, and easy-to-use biosensors look to be a very promising direction for modern biotechnologies with a high prospective for their successful commercialization [1]. Nowadays, due to permanent improvements in gadgets and user-friendly software, it has begun to be possible to adapt them for point-of-care biosensor analysis of such analytes, substituting the expensive equipment and requirement for qualified personnel [2,3]. Usually, biosensors are characterized by a limited number of target analytes to be tested as one biosensor, and are able to selectively test one specific analyte. Therefore, in order to estimate the overall data on the human state, the use of different biosensors selective to different biomarkers is required. To solve this limitation, we propose a new smartphone-adapted multianalyte biosensor platform for

the fluorescent analysis of human biomarkers and immunosuppressive drugs using PQQ- and NAD^+-dependent enzymes. The principle of the approach is based on the analysis of the brightness of photography of the sensor plate taken with a smartphone camera and processed using the free software ImageJ. The brightness of the image correlates with the fluorescence intensity of the sensor's spots which is produced by the enzymatic reduction of the fluorescent probe at long-wave UV irradiation, which correlates with the concentration of the target analyte (the specific enzymatic substrate) in the tested sample. The main goal of the investigation is to combine different enzymes selective to different analytes on the same sensor's plate to perform the direct simultaneous analysis of several drugs and/or biomarkers in the same tested sample. To simplify the analysis, we propose to analyze the specific optical output using Boolean logic gates, which gives information in a binary system as "output **1**" or "output **0**" that is equivalent to the "Yes" or "No" binary response. The proposed system was already successfully approbated for the analysis of different biomarkers and some immunosuppressive drugs in different human liquids (e.g., blood serum, urine, sweat, and saliva) [4–6]. It should be mentioned that the possibility to test the target analytes in different types of human liquids makes it more convenient for practical application because it avoids painful procedures associated with blood sampling by qualified medical personnel. This also makes it possible to increase sample collection to have continuous information about the efficacy of medication in its dynamics. We expect that the proposed multianalyte biosensor platform will give fast and accurate information to end-users (patients) directly at point-of-care, which is more convenient compared to attending clinical diagnostics centers.

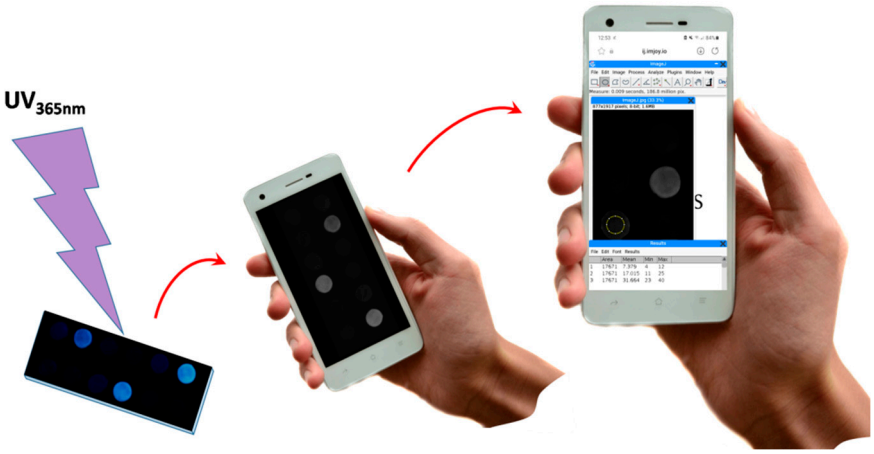
2. Background and Goal

The principle of this approach is based on the analysis of the intensity (brightness) of photography of the sensor plate taken with a smartphone camera in grayscale. The brightness of the image correlates with the fluorescence intensity of the sensor's spots which is produced by enzymatic oxidation of phenazine methosulfate or its derivative used as a fluorescence probe at UV 356 nm irradiation, where the amount of the reduced dye depends on the concentration of the target analyte (the enzymatic substrate) in the tested sample (Figure 1A,B).



(**A**)

Figure 1. *Cont.*



(**B**)

Figure 1. (**A**) Schematic presentation of the biocatalytic process generating fluorescent output proportional to the target analyte (glucose) concentration. Abbreviations: GDH—glucose dehydrogenase, Glc—glucose, GlcA—gluconic acid, PQQ and $PQQH_2$—oxidized and reduced forms of pyrroloquinoline quinone, respectively, PMS_{ox} and PMS_{red}—phenazine methosulfate oxidized and reduced forms, λ_{ex} and λ_{em}—wavelength maximum of PMS_{red} fluorescence. Figure adapted from [7] with permission. (**B**) Processing fluorescent image (sensor output) and its analysis with ImageJ software installed on a smartphone. Figure adapted from [8] with permission.

The sensor plate is composed of simple and cheap components, and the procedure of its preparation is easy and does not require any specific skills or expensive instrumentation (Figure 2).

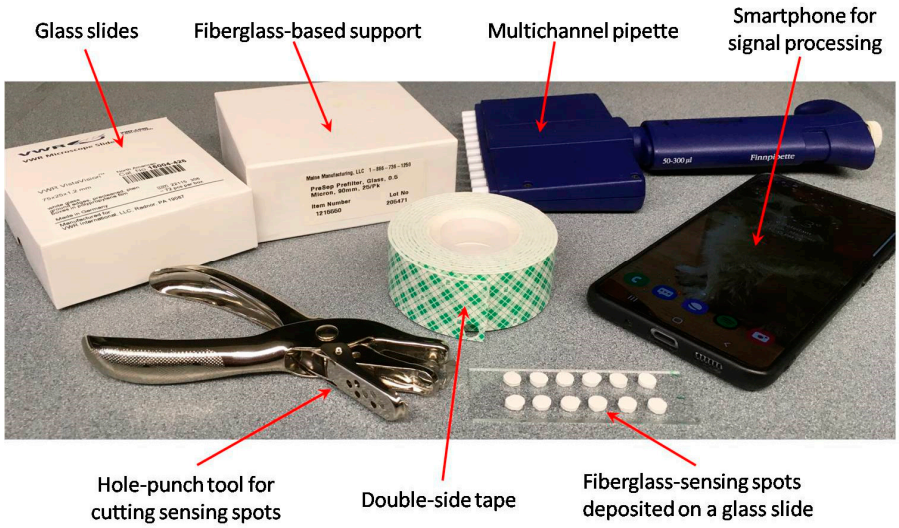


Figure 2. The components used for the biosensor preparation: VWR fiberglass filter paper, double-sided tape, microscopic glass slides with deposited fiberglass disks, an office hole-puncher for preparing the sensing disks, a multi-pipet, and a smartphone with a camera for taking photos of the biosensor platform. Figure adapted from [8] with permission.

Then, the surface of the fiberglass sheets of the sensor plate was functionalized using bioselective elements (enzymes) which were covalently immobilized on silica nanoparticles (SiO_2-NPs). The immobilization of the enzymes onto SiO_2-NPs provides two functions simultaneously: (i) high sensor stability preventing the washing out of the enzymes during the operation, and (ii) increasing the sensor sensitivity (due to amplification of fluorescence intensity) penetrating enzyme-functionalized SiO_2-NPs deep inside of the fiberglass

support and forming a multilayer structure. It should be noted that the reduced dye in contrast to its oxidized non-fluorescent form is characterized by high hydrophobicity. This characteristic of the probe additionally supports high output stability preventing leaking in buffer solutions during the washing of the sensor plate (Figure 3).

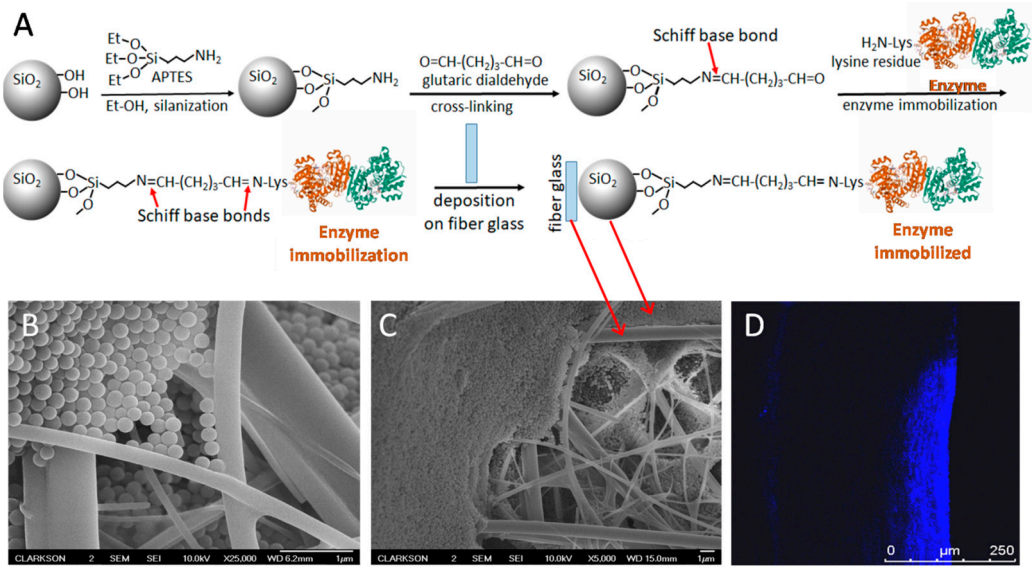


Figure 3. (**A**) The scheme of immobilization of enzymes onto SiO_2-NPs. (**B**,**C**) Scanning electron microscopy images showing the enzyme-functionalized SiO_2-NPs deposited on fiberglass support. (**D**) Microphotography of confocal microscopy visualized the distribution of enzyme-functionalized SiO_2-NPs in the sensing spot. The penetration of enzyme-functionalized SiO_2-NPs into 125 µm of the glass paper (blue color) support corresponds to app. 625 of single monolayers of SiO_2-NPs (200 nm in diameter each).

The analytical procedure of the biomarker detection by a ready-to-use sensor plate is presented in Figure 4.

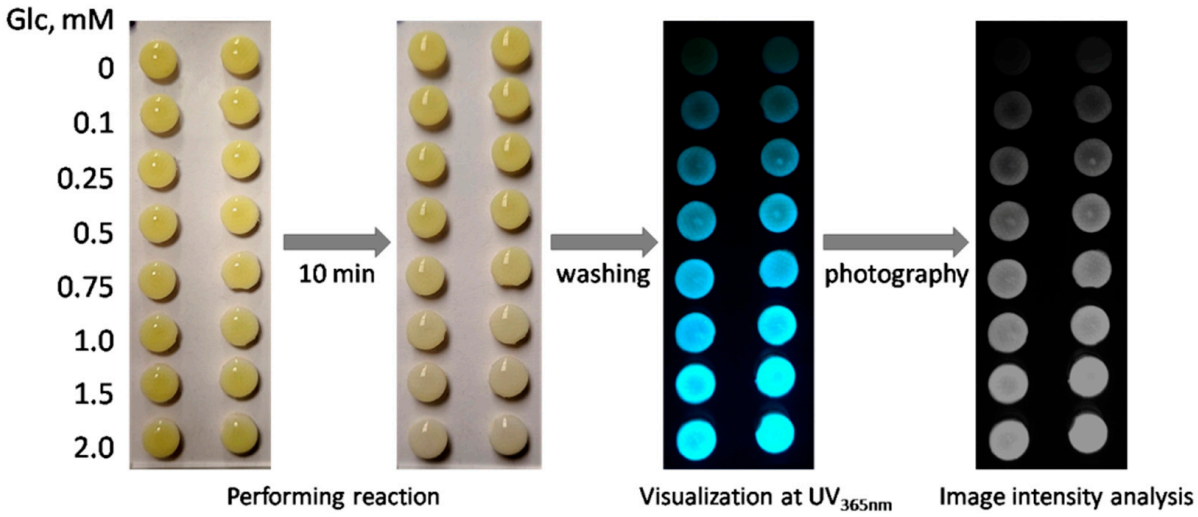


Figure 4. The sample photos of the biosensing system for the analysis of glucose (Glc) obtained at different stages of sensor analysis: as prepared, prior to application of glucose, after applying glucose at different concentrations on each sensing spot, after rinsing the sensing spots with buffer, after converting the previous photo to the gray format ready to be processed by the software "ImageJ" installed on a smartphone. Figure adapted from [8] with permission.

To take photos of the sensor plate during its irradiation by UV 356 nm, it is convenient to use the UV fluorescence analysis cabinet. However, it should be noted that, instead of a photo cabinet with an integrated UV lamp, a dark room and a cheap small lantern (with lamp 365 nm) used for an-home pet tick inspection could be used.

The proposed sensor platform is characterized by a high selectivity which depends on the selectivity characteristics of the used enzymes. The examples of the analysis selectivity for the glucose-selective biosensor based on NAD^+-dependent glucose dehydrogenase is presented in Figure 5.

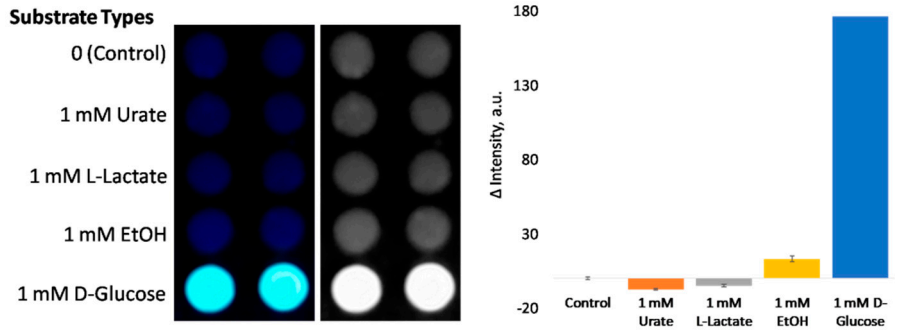


Figure 5. Selectivity test of the glucose-sensitive biosensor towards possible interferents of common blood serum compounds. Photographs show the sensing plate with the spots reacted with the target analyte glucose and with three interferents—urate, lactate, and ethanol (1 mM each)—and a bar chart showing the fluorescence intensity obtained with the target analyte glucose and with three interferents. The intensity was derived from the image as the brightness of the spots was calculated with the ImageJ software. Figure adapted from [9] with permission.

The biosensor plate storage stability depends on the stability characteristics of the used enzyme in an immobilized state. It was shown that the sensors based on different enzymes were sufficiently stable for at least of 2 months of storage at +8 °C (shelf storage). The authors believe that the storage stability of the sensor could be improved with the pretreatment of the plates with enzyme stabilizing agents, using freeze drying, vacuum packing, etc.

The proposed sensor system was approbated for the analysis of human biomarkers and some immunosuppressive drugs in different human liquids (blood serum, saliva, sweat, urine). The list of tested analytes is presented in Table 1 with indicated corresponding references where the detailed procedures of assay are described.

Table 1. The list of analytes tested by means of the developed multianalyte biosensor platform.

PQQ-Dependent GDH (Including Chimeric Forms):		NAD^+-Dependent Dehydrogenases:	
1. Glucose	[7]	1. Phenylalanine	[6]
2. α-Amylase	[5]	2. Glucose	[9]
3. Cyclosporine A	[4]	3. Lactate	-
4. Tacrolimus (FK 506)	[4]	4. Pyruvate	-
5. Rapamycin	[10]	5. Alcohol	[9]
6. Methotrexate	[11]	-----	

The proposed sensor platform could be used for precision quantitative analysis of a single (or several) analytes or used for the simultaneous qualitative multianalyte assay of many of them.

For the simultaneous multianalyte analysis, the most convenient seems to be a binary system used in Boolean logic gates where a logical operation uses one or more binary inputs to produce a single binary output [12]. In this case, the surplus amount of the analyte will

be represented as fluorescent output **1** when its amount in normal concentration or below the norm produces the fluorescent output **0** or the signal is significantly lower than output **1**. The sample of using the proposed sensor platform via Boolean logic gates is presented in Figure 6.

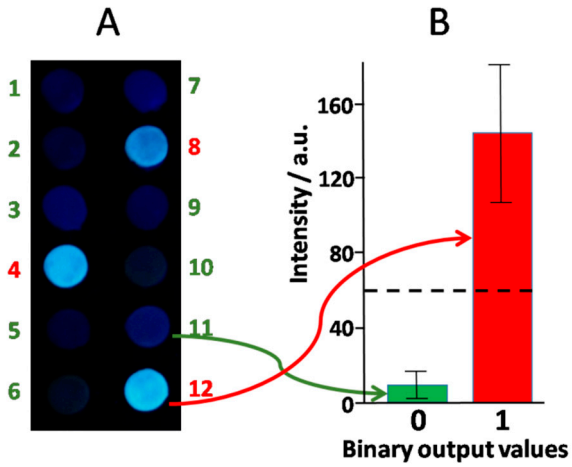


Figure 6. The sample of using the proposed fluorescent biosensor system for multianalyte analysis using Boolean logic gates. Each pair of sensing spots is modified with different enzymes selective to a unique analyte. When all the sensor spots are treated with the same tested sample (e.g., human serum from one patient) the increased amount of some biomarkers will provide the intensive spot's fluorescence (output **1**) (see intensive blue spots in (**A**)) when their normal level will be taken as "no output" (output **0**) (see dark blue/weakly visible spots in (**A**)). (**B**) The bar chart shows the fluorescence intensity of the spots corresponding to the logic outputs **0** and **1** derived from the images by calculating the image brightness using the ImageJ software.

The simultaneous combination of output **1** from spots sensitive to glucose, lactate, cholesterol, and/or glycerol will indicate a high probability of diabetes in the tested body or some adiposity-related metabolic dysfunctions. The combination of other biomarkers from the list in Table 1 will predict other health issues or the current human physiological states and this is the aim of the ongoing work.

3. Summary and Outlook

The proposed multianalyte optical biosensor platform is characterized by high selectivity, sensitivity, and sufficient storage stability. Moreover, it is cheap and easy to prepare and use. It was tested for highly sensitive accurate analysis of such human biomarkers as α-amylase, phenylalanine, glucose, lactate/pyruvate, alcohol, and some immunosuppressive drugs such as cyclosporine A, tacrolimus, methotrexate, and rapamycin. The possibility to analyze the target analytes in different human bioliquids (e.g., blood serum, urine, sweat, and saliva) was demonstrated, thus allowing sample collections from patients, avoiding painful puncturing procedures. Due to the possibility of fast simultaneous analysis of several biomarkers/drugs, this system could be very helpful for point-of-care use, substituting expensive and time-consuming routine techniques of clinical diagnostics labs.

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Conflicts of Interest: The authors declare no conflict of interest.

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