A preclinical biosensor for detecting phenylalanine photometrically in plasma and whole blood samples

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ABSTRACT.

Phenylketonuria (PKU) is a metabolic disease resulting from a deficiency in the enzyme phenylalanine hydroxylase, increasing L-Phenylalanine (L-Phe) values in the blood and consequently in the brain. If untreated, PKU leads to neurological damage, which can be prevented by following a diet low in L-Phe. Thus, early detection of PKU in newborns is essential. The disease's screening and monitoring are centralized in reference centers, which require specialized equipment. However, using these techniques, sample treatment is required before the analysis, and trained personnel must perform and interpret the results. In this work, we present an enzyme-based-photometric strategy to measure blood L-Phe. An enzymatic mixture, selective for L-Phe, is immobilized on an UV transparent well, and the amount of consumed co-factor is monitored at 340 nm. Standard plasma and whole blood samples were chosen to pre-validate the sensor. The samples were spiked with an increasing amount of L-Phe, accurately discriminating between physiological and pathological L-Phe concentrations. The strategy can be easily extended to analyzing other samples, such as urine or sweat. The proposed photometric system allows to analyze up to 16 samples simultaneously within a matter of hours. The measurements are relatively fast, versatile, cost-effective, and easy to carry out.

Keywords: Phenylketonuria; L-phenylalanine; photometric biosensor; phenylalanine dehydrogenase.

Introduction

Phenylketonuria (PKU) is a genetic metabolic disease caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH), leading to high blood L-Phenylalanine (L-Phe) levels, which can cross the blood-brain barrier and accumulate in the brain. This phenomenon has been related to different pathomechanisms. Among others, white matter structural changes have been demonstrated in PKU. Also, high L-Phe levels have been correlated to low availability of neurotransmitter precursors (tyrosine and tryptophan) in the central nervous system, leading to decreased biosynthesis of dopamine and serotonin(Bilder et al. 2016). The main consequences of having high brain L-Phe levels for long periods are that they induce IQ loss, lead to cognitive and attention issues and behavior problems, among other neurological complications.(Romani et al. 2017) Implementation of a specialized diet during the newborn period averts the complications associate to the disease.

PKU has a varying prevalence worldwide, with a mean of 0.01% in the US and Europe, higher incidences in countries such as Turkey and Ireland, and lower in Finland.(Paul and Brosco 2013) The current neonatal screening approach(Paul and Brosco 2013; van Wegberg et al. 2017) generally consists of using tandem mass spectrometry (MS/MS) to detect, among other diseases, all potential cases related to high levels of L-Phe, which include those with PAH deficiency (the most common cause of hyperphenylalanemia), those with tetrahydrobiopterin (BH_4) metabolism defects, and those with the recently reported hyperphenylalaninemia due to mutation at the *DNAJC12* gene. This neonatal screening test is done on dried blood on filter paper specimens with a L-Phe cut-off value of 120 μmol/L, and are performed, like most neonatal screening tests during the 24-48 hours of life. These initial screening results are communicated to the newborn's family during the first week of life, after which a definitive differential diagnosis of the different diseases leading to hyperphenylalaninemia is (ideally) given during the first month of life. The routine application of next-generation sequencing (NGS) techniques is accelerating this process. While this early diagnostic activity is fully implemented in developed countries through national screening programs, the same is not standard practice in most developing countries. Therefore, there is an unmet need for an alternative, more affordable analytical L-Phe detection strategy in developing countries.

Once PKU has been detected and diagnosed, L-Phe levels must be continuously monitored for life during the imposed low L-Phe diet therapy, which is the most common treatment against PKU. Routine phenylalanine monitoring depends on a patient's age, varying from weekly frequency in infants to fortnightly or monthly frequency in children < 12 years of age and adolescents/adults. Regarding recommended L-Phe values for follow-up checks, they vary slightly between American and European guidelines. However, it is generally agreed that L-Phe values below 360 μmol/L for children less than 12 years of age and women before conception and pregnancy, or below 600 umol/L for adolescents/adults, are considered safe (European guidelines(Lowe, DeLuca, and Arnold 2020)). Furthermore, L-Phe being an essential amino acid for average growth and development, values < 120 μmol/L are not recommended for PKU patient in long-term follow-ups. Alternative therapy to the low L-Phe diet consists of administering BH_4(on behalf of the International Working Group on Neurotransmitter related Disorders (iNTD) et al. 2020), a PAH co-factor, to patients who are candidates for this therapy. However, only a tiny percentage of PKU cases have shown to respond positively to this treatment. A few alternative therapies have recently received approval by the Food and Drug Administration (FDA) and show promise for improving the quality of life of PKU patients. The first FDA-approved (on May 24, 2018) enzyme substitution therapy for (adult) patients with PKU

is Pegvaliase, a pegylated Phenylalanine ammonia-lyase (PAL) enzyme that converts phenylalanine to ammonia and trans-cinnamic acid. This therapy is currently on the market under the brand name “Palynziq” (Biomarin) and is recommended for patients with documented blood L-Phe >600 μmol/L who have failed existing management strategies. However, the administration of Pegvaliase does come with a risk of anaphylaxis and is therefore available through the Risk Evaluation and Mitigation Strategy (REMS) program(Hydery and Coppenrath 2019). Other therapeutic approaches for PKU are currently under investigation(Charbonneau et al. 2020) and may be found at an available online database (e.g. www.clinicaltrials.gov).

Many studies aimed at developing new PKU monitoring strategies, relying on several different approaches, have been published. These include enzyme-based(Gubica et al. 2015; Mangombo et al. 2013; Naghib, Rabiee, Omidinia, and Khoshkenar 2012; Naghib, Rabiee, Omidinia, Khoshkenara, et al. 2012; Naghib, Rabiee, and Omidinia 2014a, 2014b; Omidinia, Khanehzar, et al. 2013; Omidinia, Shadjou, and Hasanzadeh 2014b; Villalonga et al. 2007, 2008; Weiss et al. 2007; Zhuo Wang et al. 2005), aptamer-based (Idili et al. 2021), (Omidinia, Shadjou, and Hasanzadeh 2014a) and nanomaterials(Hasanzadeh et al. 2009, 2012; Hasanzadeh, Shadjou, and Omidinia 2013; Omidinia, Shadjou, and Hasanzadeh 2013; Wu et al. 2017) based electrochemical sensors, as well as immune sensors,(Kubota, Mizukoshi, and Miyano 2013) quartz crystal microbalance (QCM),(Cho et al. 2017; Emir Diltemiz et al. 2017; Mirmohseni, Shojaei, and Farbodi 2008) extended field gate field effect transistor(Iskierko et al. 2017) and photometric-based strategies. Some of the principle challenges facing the development of an effective biosensor for PKU monitoring include (1) having a linear range which covers the 120 μmol/L and 360 μmol/L key threshold values for L-Phe described previously, (2) being able to detect L-Phe in patient-derived samples with low chemical interference (e.g. from other amino acids), (3) a design that is intuitive and user-friendly and (4) a fast detection time.

Despite the broad interest, an easy, low-cost and fast L-Phe analytical system has yet to be developed. In this work, we provide such a method based on the indirect photometric detection of L-Phe, which uses a commercially available phenylalanine dehydrogenase (PDH) enzyme. We chose PDH over PAL to adapt our biosensor system to detect analytes using other dehydrogenases (Lactate-, tyrosine-, glucose-, pyruvate-, etc dehydrogenase). The photometric detection system was designed to be compatible with UV-transparent microplates. The PDH enzyme, mixed with stabilizing components, is immobilized at the bottom of the microplate well using a Nafion membrane. This strategy minimizes the preparation for the final end-user as the modified microplate can easily be stored at 4°C and used when measurements are needed. The end-user only has to prepare the measurement solution containing the sample, buffer and co-factor. The photometric system described in this work was fully characterized, with the best working conditions, long-term storage, the study of possible interferents and initial validation in plasma samples (the sensor output correlates linearly with the values of the hospital, determined using a validated protocol) and whole blood samples. The results described here offer a promising solution for PKU L-Phe monitoring.

Materials and methods

All chemicals were used as received without any further purification. Glycine Buffer pH 10.5 (prepared with Glycine ReagentPlus, ≥99% (HPLC); Sigma Aldrich), phosphate buffer (PB) pH 7.4 (pH adjusted with Potassium Phosphate monobasic ACS reagent ≥99%, Sigma Aldrich), L-Phe BioUltra ≥ 99% (NT); Sigma Aldrich, β-NAD; Thermo Fischer Scientific, L-Phenylalanine Dehydrogenase from *Sporosarcina sp*. (PDH, 50 U); Sigma Aldrich, Bovine Serum Albumin (BSA) lyophilized powder; Sigma Aldrich, 0.5% Nafion 117 solution; Sigma Aldrich. UV transparent microplates were used. The SYNERGY HTX multi-mode microplate reader (Biotek) was used for the absorption measurements.

Plasma and whole blood samples were provided by the Hospital "San Joan de Deu", the reference center for PKU follow-up in Catalonia. Both plasma and whole blood samples were prepared from quality control materials and healthy volunteers, spiked with different amounts of L-Phenylalanine (n=106 in total for plasma; 61 samples were used to establish the optimal technical conditions of the biosensor, and then, the remaining 45 were used to be compared with those analyzed in HSJD and n=5 for whole blood). The range of concentrations was between 49 -1800 µM, covering the range of standard and very high phenylalanine values. L-phenylalanine values were determined by a fully validated reference method (liquid chromatography/tandem mass spectrometry) as previously reported.(Casado et al. 2018) This method has been accredited by the Spanish accreditation agency (ENAC, ISO15189 norm), and our laboratory participates in the external quality control scheme ERNDIM for amino acids (data available on request). For three L-Phe values (300 µM, 450 µM and 700 µM), ten additional measurements in triplicate were performed (30 samples analyzed as a whole), and subsequently analyzed employing an f-test and a two-tailed student *t*-test to search for statistical differences among these groups. For the initial validation of the biosensor, all samples were analyzed in a blinded way. This study was approved by the Ethical Committee of Hospital Sant Joan de Déu (project number DTS18/00075). All methods were carried out in accordance with the 2013 revised Helsinki Declaration of 1964. Blood donor volunteers were recruited from Hospital Sant Joan de Déu laboratory staff. Informed consent was obtained from all subjects, and informed consent is available on request.

Dropcasted solution.

Prior to modifying the biosensor microplates, a solution of phosphate buffer (pH 7.4) containing BSA (20% V/V), PDH (8.4 % V/V) and 0.5% of Nafion (2.7% V/V) was prepared. An amount of 7 µL of this solution was then drop cast to each test well, which constituted 45 wells of the 96-well plate, and left to dry overnight at 4^{o}C. The other wells in the plate were left unmodified to measure the blank negative controls. For initial measurements in glycine buffer, the final concentrations of BSA and PDH were 14 µM and 1 U/cm^2, respectively. In plasma, BSA and PDH concentrations were optimized to 28 µM and 3 U/cm^2, respectively (*vide infra*). For measurements performed in whole blood, the PDH concentration was increased to 6 U/cm^2, while BSA concentration remained fixed at 28 µM.

Measurement protocol.

For the measurements conducted in buffer, 200 µL of 250 mM glycine buffer (pH 10.5), with 6 mM NAD^+ and variable concentrations of L-Phe were added to each of the blank wells. After running the blank control measurements, 180 µL of this measurement buffer were added to the test wells. For measurements performed in plasma, the L-Phe was omitted from the measurement buffer, and 5 µL of the plasma samples were added to the same buffer. Blank

controls and test experiments were performed using the same procedure as those in the buffer. For measurements in whole blood, 1 µL of a 1000 X dilution of the whole blood samples were added to the measurement buffer, which contained 6 mM NAD^+. Measurements were then conducted as previously described.

Sensor preparation.

The optical biosensor was fabricated according to the steps laid out in Figure 1 A. First, the wells were modified with the PDH enzyme, mixed in a solution along with BSA and Nafion (see Figure 1 B), dropcasted into the wells and left to dry. BSA was added to the mixture to stabilize PDH, and the Nafion polymer served to fix the enzyme mixture onto the bottom of the well, limiting desorption. PDH activity was then assayed by adding a solution of NAD^+ and L-Phe dissolved in glycine buffer at pH 10.5, and measuring the rate of change in absorbance at 340 nm over time, corresponding to the absorbance peak of reduced co-factor, NADH (see the corresponding chemical reaction in Figure 1 D). Indeed, the interconversion of NAD^+ with NADH is a commonly used technique to evaluate the catalytic activity of dehydrogenases, as the UV absorption spectra of the reduced form differ from that of the oxidized form by the presence of a second peak at 340 nm in the UV range (Gloster and Harris 1962). For a fixed concentration of NAD^+, the initial slope of the Abs_{340} response over time increased with increasing L-Phe concentration.

(insert Figure 1 here)

Statistical analysis

The statistical analysis was done by initially performing a F-test to evaluate if equal or unequal variances need to be considered for the t-test analysis. Once it was established that both populations presented equal variances, a two-tail t-test was performed considering a cut-off p-value of 0.05, rejecting the null hypothesis that both populations have no significant difference (presenting a mean difference of zero) when below this value.

Bill of material (BOM)

The total cost is provided for measurements in the whole blood. We recommend peforming triplicates of the measurements for a more reliable output. Therefore, the cost per patient is considered from the cost of the measurement done in triplicate .

The cost per patient is calculated as follows: we consider the cost of the amount of enzyme per well 0.0196U/well (PDH 437€ for 50U), which gives 0.1713€/well which gives 8.22€/microplate (48 wells of the test). The amount of co-factor NAD^+ (72.2€/g) we use is 42.12 mg/microplate, giving 2.97€/microplate. For the BSA (50g costs 181.8€) we make 24 mg/90 ul and use 18 uL/well, giving thus 0.84€/microplate. The Nafion (25 ml/176.5€) is diluted 10X, and 18 ul is added, which corresponds to 0.608€/microplate. The final estimate is 13.64€/microplate, including the price of the microplate itself, which corresponds to 0.28€/measurement (0.85€ in triplicate). For the optimized conditions in plasma, we use 2X BSA (2X0.84 €) + 3X PDH (3X8.22€) more. The described calculation gives 30.02€ per microplate, 0.64€ per measurements and 1.92€ per triplicate and therefore per patient. For whole blood, we use 2X BSA (2X0.84 €) + 9X PDH (3X8.22€) more and therefore, the price per patient is 4.95€.

Results

The sensor is characterized by varying many parameters to find the optimal working conditions of the PKU biosensor (Figure S1). The optimization considers high-performance and cost-efficiency. For the initial characterization, we used a fixed concentration PDH 1 U/cm^2., BSA 14 µM, NAD^+ 6mM and T=30°C and pH=10.5. Each variable was varied independently, see Figure S1.

First, for the dropcasting mixture used to modify the wells, it was found that the signal output was highest at the initial pH of 10.5 (Figure S1 A), then NAD^+ was varied, and 6mM was chosen (Figure S1 B). The best temperature was also shown to be 30°C (Figure S1 C). These were the conditions used and chosen to do the initial calibration tests and interference analysis in the buffer. For plasma analysis, the BSA and PDH amount were optimized (Figure S1 D) to avoid any matrix effect when performing tests on plasma. This optimization showed that 3 U/cm^2 of PDH needed to be immobilized in each well to provide the best performance. BSA was added to stabilize PDH, preventing it from denaturing during the immobilization process. Upon increase of BSA concentration in the dropcasting mixture, the overall activity of PDH was improved (Figure S1 D), and was optimal at 28 µM.

To characterize the enzymatic activity of the biosensor in the newly optimized conditions, the initial slopes from the biosensor assays, conducted at different concentrations of L-Phe (in buffer), were converted to the amount of generated NADH per second using Beer-Lambert Law and fitted with a Michaelis–Menten equation (Figure 2 A). An apparent K_M of 1,683.021 µM was obtained from the Michaelis–Menten fit, and the reaction rate of the biosensor reached saturation for approximately 5 mM of L-Phe substrate, a value outside the physiological and pathological ranges. Figure 2 B shows the linear range of the Michaelis–Menten graph in Figure 2 A, which is between 30 µM and 1 mM. Good linearity was observed (R^2=0.961) covering both the safe range (< 360 µmol/L) and those corresponding to mild to severe hyperphenylalaninemia (from 360 to higher than 1000 µmol/L), demonstrating the potential adequateness of the photometric biosensor for PKU monitoring. Moreover, the sensitivity and limit of detection (LOD) of the biosensor could be determined from the linear fit of the data in Figure 2 B (5.86 x10^{-5} Abs.s^{-1} and 1.03 µM, respectively). The LOD and additional parameters of interest are indicated in Table 1, which also compares and contrasts the results from the biosensor described in this work with similar devices reported in the literature.

(insert Figure 2 + Table 1)

The selectivity and shelf-life of the biosensor are evaluated employing the initial conditions. PDH's specific reactivity towards L-Phe is often hindered by the presence of interferents, such as tyrosine (see Table 1). Therefore, the PKU biosensor's activity towards several different (potentially interferent) amino acids was tested (Figure 2 C). The PKU biosensor presented high selectivity to the L-Phe substrate, with minimal interference from arginine, glutamine, tryptophan, cysteine and tyrosine (Figure 2 C). Each interferent was tested at relevant physiological concentrations present in plasma(Canepa et al. 2002), bearing in mind that these reference values may vary slightly among laboratories. The stability of the biosensor over time is displayed in the inset of Figure 2 C, where it is shown that, if stored at 4 °C, the biosensing capability is stable over the period of 4 weeks.

The PKU biosensor was thus optimized for L-Phe quantification in pH 10.5 buffer and could undergo validation on anonymous plasma samples in a mixture containing quality control materials at different concentrations, provided by the Hospital "San Joan de Deu" (HSJD). As

previously indicated, to avoid any matrix effect from the plasma samples, the measuring conditions were adjusted. The PKU biosensor was operated using the following optimized parameters in the buffer: 6 mM NAD^+, 30 ^oC, pH 10.5, 3 U/cm^2 of PDH and 28 µM of BSA. We also reduced the sampling volume to 2.5% v/v, which again helps avoid any matrix effect, see methods section. No effect from possible interferents was observed under these conditions; see Figure 2D. A single-blind test with our L-Phe biosensor on the plasma samples was then carried out, with no prior knowledge of the samples' L-Phe concentration, determined by the hospital using MS/MS. In Figure 3 A, a clear linear correlation is found between our biosensor output values with the hospital values provided at a later stage, supporting the robustness of our biosensor and providing a calibration curve that allows converting the units from NADH/s to µM. In Figure 3B, by applying the correlation described in Figure 3A, the biosensor was used to detect a range of L-Phe concentrations and classify them into healthy (green), mildly elevated (orange), high (red) and significantly elevated (burgundy) groups by using corresponding threshold values of [L-Phe] < 120 µM, 120 µM < [L-Phe] < 360 µM, 360 µM < [L-Phe] < 1200 µM and 1200 µM < [L-Phe], respectively.

The current preclinical study is the first stepping stone towards a full clinical validation of the proposed biosensor in the hospital setting. These results enable our biosensor to be potentially used for monitoring the L-Phe levels of pre-diagnosed PKU patients. Considering that L-Phe values below 360 µM are considered safe for children younger than 12 years old and that those above 600 µM are higher than those recommended for PKU patients older than 12 years, we included a plot of measurements done on 30 plasma samples, measured in triplicate, 10 with 300 µM [L-Phe], 10 with 450 µM [L-Phe], and 10 with 700 µM [L-Phe] (Figure 4A). The confidence interval for these measurements, was obtained by performing a *t*-test analysis. As can be observed, the calculated p values are below p=0.05 and therefore rejects the null hypothesis (mean difference between both populations is zero) and therefore indicating that the 300 µM, 450 µM and 700 µM [L-Phe] are statistically distinguishable between each other. The presented data shows that the device can alert when L-Phe concentration values are unsafe, and by extension when the diet or BH_4 therapies need to be readjusted.

To complete our preclinical study, the biosensor was tested against whole blood samples. In this case, to achieve a good sensing performance of the L-Phe biosensor, an even more significant dilution was required (5000 times more diluted compared to the blood plasma) and an increase in enzyme concentration (9 U/cm^2). A total of five anonymous whole blood samples were blind tested in a similar way to the tests done on plasma. The obtained correlation function is presented in Figure 4B.

Discussion

Overall, a highly sensitive and stable L-Phe photometric biosensor for plasma samples is described.

The overview presented in Table 1 shows that our proposed photometric setup has many benefits over others reported in the literature. Our PKU sensor is easily scalable, cost-effective, and could analyze L-Phe in different biological samples if needed. Moreover, multi-analyte detection would be possible through the immobilization of other dehydrogenases (e.g. glucose - glucose-6-phosphate dehydrogenase, aldehyde - Aldehyde dehydrogenases, etc.), and the measurements are relatively fast compared to traditional methods. The system is simple, easy to prepare (minimum processing is needed), has long-term stability (only ref(Pijanowska and Remiszewska 2006)presents results for the durability of their sensor) and is fully characterized.

The L-Phe biosensor has been pre-validated on plasma and whole blood samples provided and analyzed by the HSJD. The obtained results show that the presented sensor design is highly recommended for determining L-Phe levels in blood plasma (only a few work showed results in the same matrix previously (Brunhuber et al. 2000; Hummel, Schütte, and Kula 1988; Johnson and Morrison 1970). Our preliminary data also shows the possibility of adapting the sensor to measure L-Phe in whole blood (only (Arakawa et al. 2011) in Table 1 describes a similar capacity) potentially allowing an efficient L-Phe monitoring system for use in at-home settings or outside reference centers.

To highlight the potential of our biosensor and to strengthen the data shown in Figure 3, 30 additional blood plasma sample measurements were done under the same conditions (i.e blind measurement and posterior validation with the hospital values), with the results shown in the plot of the supporting information in Figure S2. Significant statistical differences were observed among the different concentrations chosen, supporting the future usefulness of the biosensor, once fully validate, to make clinical decisions regarding treatment modification.

The results on whole blood samples are of great importance, as measuring whole blood samples is not straightforward due to the high complexity of this sample. Moreover, this provides an excellent alternative to pre-existing diagnostic L-Phe analysis systems. However, it should be noted that the need to increase the enzyme concentration raises the overall cost of the biosensor considerably concerning the tests on plasma. Strategies to mitigate this increase in the price of the biosensor while improving its sensitivity to L-Phe in whole blood will be further explored in future work. Nevertheless, the cost of the test per patient on blood plasma is below 2 euros and for whole blood samples remains below 5 euros (see the methods section for a detailed bill of materials), which makes our sensor a desirable option for developing countries.

Limitations and future directions.

This is a preclinical study, the results of which need to be thoroughly tested employing a "validation of a medical device" study. The first step will be to fabricate a user-friendly point-of-care device used in the hospital laboratory, which should be compared with the standard method for plasma and whole blood L-Phe monitoring during PKU patient follow-up. The system needs to be robust since the next step will be designing an at-home device for L-Phe monitoring. As previously mentioned, according to the PKU guidelines, L-Phe monitoring frequency varies between weekly and monthly, depending on the patient's age. The possibility of analyzing L-Phe values at home with a higher frequency (as occurs in other diseases, such as diabetes) can improve our knowledge about the clinical outcome of PKU patients concerning the fluctuation of blood L-Phe over time.

Conclusions

Overall, a highly sensitive and stable L-Phe photometric biosensor for plasma is described. Its fast measurement and analysis can allow in the future, after extensive clinical validation, for more frequent analyses, which is crucial for PKU monitoring and understanding the fluctuations of L-Phe levels over time. The device is potentially easily adaptable for analyzing other biological fluids (urine or sweat), thus reducing invasiveness. The immobilization of the enzyme at the bottom of the microplate wells is highly convenient and provides excellent versatility to other enzymatic reactions using NAD^+ as a co-factor (namely dehydrogenases). This could lead to multi-biosensing photometric platforms, on the same microplate, by parallel analysis of the absorbance kinetics at 340 nm.

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Author contribution statement

PEDSR, AR, and SS conceived the idea, designed and lead the experiments. PEDSR, MV and AR did the characterization on the buffer. PEDSR and MV realized the experiments and optimization for the detection of plasma and whole blood samples. RA collected and instructed on the correct manipulation of the hospital provided samples. All authors analyzed and discussed the data. All authors contributed to the paper writing and agreed with the final version.

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Figure captions

Figure 1: Steps of biosensor fabrication, assay and analysis. Created with BioRender.com

Figure 2. Characterisation of biosensor in buffer. A) Michaelis–Menten fit of experimentally determined reaction rates for PDH-mediated L-Phe turnover in buffer. *Inset:* Calibration plot of [L-Phe] in PBS 1X pH 7.4 mixed in the measurement buffer. The obtained calibration equation is $[NADH]/_{sec}\left(\mu M/_{s}\right) = 0.025\left(1/_{s}\right) \cdot [L-Phe](\mu M) + 1.807\left(\mu M/_{s}\right)$. B) Interference study with plasma optimized conditions (PDH= 3 U/cm^2, BSA= 28 µM, NAD$^+$= 6 mM, T=30ºC, and pH=10.5) *Inset:* Shelf lifetime of the photometric platform for L-Phe detection measured in the presence of L-Phe 500 µM.

Figure 3. Preclinical validation of the photometric platform for L-Phe sensing on 15 plasma samples. Discrimination between average and high L-Phe values was observed.In figure A) the correlation plot with values provided by the hospital are shown. The obtained calibration equation is $[NADH]/_{sec}\left(\mu M/_{s}\right) = 0.016\left(1/_{s}\right) \cdot [L-Phe]_{Hospital}(\mu M) + 5.419\left(\mu M/_{s}\right)$. In figure B) the corresponding analysis, discriminating among the most importan [L-Phe] levels is shown.

Figure 4. Preclinical validation of the photometric platform for L-Phe sensing on 30 plasma samples measured in triplicate and 5 whole blood samples. Discrimination between the 3 sets of L-Phe values was observed * stands for p<0.05 and *** for p<0.001. A) plasma L-Phe values of 300 µM, 450 µM and 700 µM with results of *t*-test analysis. B) The same methodology as for the the blood plasma was applied for a set of whole blood samples provided by HSJD. The obtained calibration equation is $[NADH]/_{sec}\left(\mu M/_{s}\right) = 0.008\left(1/_{s}\right) \cdot [L-Phe]_{Hospital}(\mu M) + 0.6416\left(\mu M/_{s}\right)$.

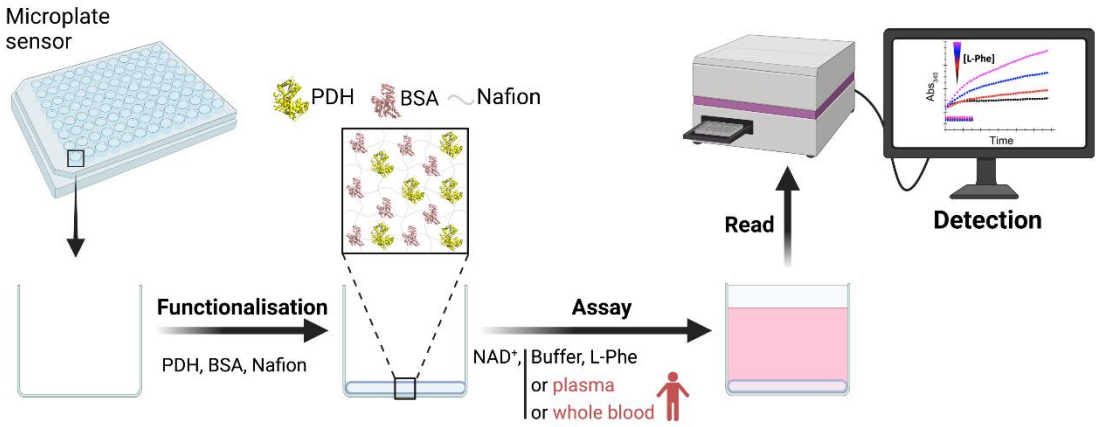
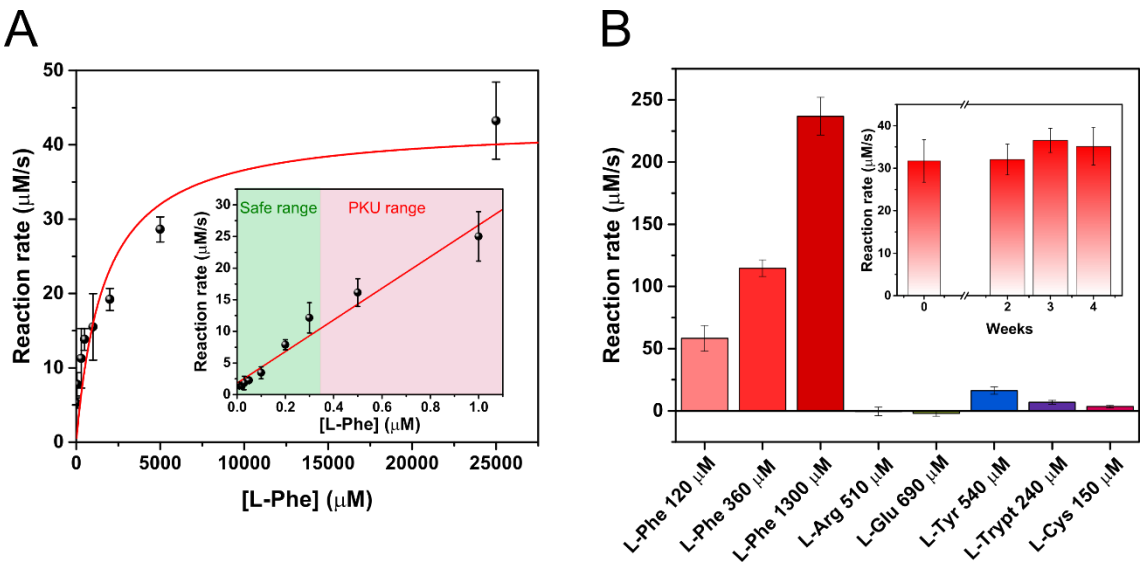

Microplate
sensor
PDH
BSA
Nafion
Functionalisation
PDH, BSA, Nafion
Assay
NAD+, Buffer, L-Phe
or plasma
or whole blood
Read
[L-Phe]
Time
Detection

Figure 1



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572 **Figure 2**

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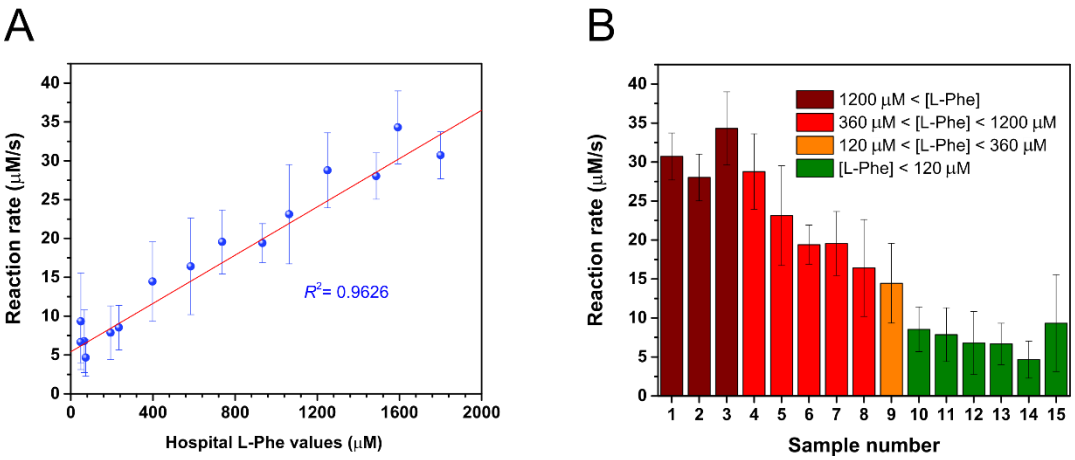

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575 **Figure 3**

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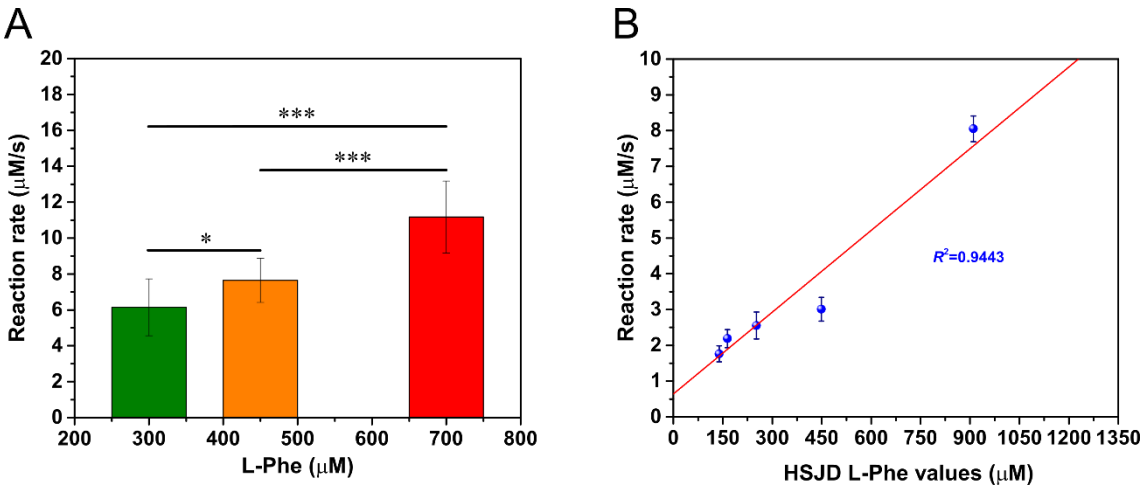

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578 **Figure 4**

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Sensing material	Detect. principle	Device type	LOD (µM)	Linear range (µM)	Real sample	Validation	Temp (°C)	Buffer	Test time (min)	Interferents tested	Ref
PDH Brevibacterium-in liquid	NADH absorb. (340 nm)	Slope	10-20	10-300	-	Autom. AA	30	0.1 M glycine buffer, pH 10.7	1	Tyr, PP	(Hummel et al. 1988)
PDH Rhodococcus - in liquid	NADH absorb. (340 nm)	Endpoint	-	30-2000	Plasma	Autom AA	21	glycine buffer, pH 10.8	10	PP, tyr, AA	(Wendel, Koppelkamm, and Hummel 1991)
PDH Rhodococcus - in liquid	NADH -> formazan (492 nm)	kinetic	1.5 - 3	30-1200	Plasma	Autom. AA	21	K-phosphate + triethanolamine + Triton	30	PP, tyr, AA, pyruvate	(Wendel, Hummel, and Langenbeck 1989)
PDH Rhodococcus - in liquid	NADH absorb. (340 nm)	Endpoint	-	-	-	Colorim. (pH indicators)	RT	glycine buffer pH 10.56	90	-	(Pijanowska and Remiszewska 2006)
PDH Thermoactinomyces intermedius – immobilized on filter membrane	NADH fluo emiss (493 nm)	slope	>5	20-10000	-	-	25	PB, pH8.0, 80 mM	5	Gly, Tyr, Leu, Val, Isoleu, Methio	(Arakawa et al. 2011)
PDH Sporosarcina – immobilized on UV transparent well	NADH absorb. (340 nm)	slope	1.03	30-1000	Plasma, whole blood	Plasma, whole blood HPLC	30	glycine buffer pH 10.56	30	Arg, D-Trypt, Tyr, Cys, Glut	This work
PDH Sporosarcina- in liquid	NADH -> formazan (510 nm)	slope	69 (LOQ)	0-9000	Plasma	AAA, HPLC, MS/MS	-	K-phosphate + triethanolamine pH 8.6	1	Tyr	(De Silva, Oldham, and May 2010)
PDH – immobilized on paper	NADH -> formazan (340 nm)	Endpoint	0.3	Only 4 points	Plasma	Phe assay	25	50 mM bis-tris propane pH 9.3	10-20	-	(Thiessen et al. 2014)
PDH- immobilized on paper	NADH -> formazan (340 nm)	-	-	60-300	Blood	-	25	blood	6+2	-	(Robinson et al. 2016)

PAL- immobilized on paper	Phe -> NH_3 (pH indicator) - immobilized	-	20	20-3000	Urine	MS–MS	35	Na-PB + NaOH	20	-	(Messina et al. 2017)
MNPs-MIP	Raman (532 nm)	-	0.4 µg/mL	10-1000 µg/mL	Urine	-	-	PBS	20	Tyr, L-DOPA, dopamine	(Hsu et al. 2015)
WO_3-NPs	Photochromic WO_3 (775 nm)	-	1	10-1000	-						(Tanaka, Adachi, and Yamazaki 2013)

581 **Table 1: Comparison with Literature**