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# Monitoring Metabolites Using an NAD(P)H-sensitive Polymer Dot and a Metabolite-Specific Enzyme

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***Abstract:*** *We introduce an NAD(P)H-sensitive polymer dot (Pdot) biosensor for point-of-care monitoring of metabolites. The Pdot is combined with a metabolite-specific NAD(P)H-dependent enzyme that catalyzes the oxidation of the metabolite, generating NAD(P)H. Upon UV illumination, the NAD(P)H quenches the fluorescence emission of Pdot at 627 nm via electron transfer, and also fluoresces at 458 nm, resulting in a shift from red to blue emission at higher NAD(P)H concentrations. Metabolite concentration is quantified ratiometrically-based on the ratio of blue-to-red channel emission intensities, with a digital camera-with high sensitivity and specificity. We demonstrate phenylalanine biosensing in human plasma for a phenylketonuria screening test, quantifying several other disease-related metabolites (lactate, glucose, glutamate, and β-hydroxybutyrate), and a paper-based assay with smartphore imaging for point-of-care use.*

## Introduction

Metabolites are participants in biochemical reactions that support essential biological processes such as energy conversion, biomacromolecule formation, epigenetic modifications, and cell signaling.[1,2] Quantitation of metabolites in patient bodily fluids is critical in the diagnosis and management of diseases and other medical conditions.[3,4] For example, phenylalanine is measured to assess phenylketonuria,[5] glucose to manage diabetes,[6] leucine to monitor maple syrup urine disease,[7] lactic acid to assess tissue oxygenation,[8] tyrosine to detect tyrosinemia,[9] glutamate to assess ischemic strokes,[10] β-hydroxybutyrate to manage hyperketonemia,[11] and α-ketoglutarate to monitor nonalcoholic fatty liver disease.[12] However, the vast majority of metabolites cannot be quantified at the point of care, and there is a need for simple, low-cost, rapid metabolite assays to improve disease diagnosis, healthcare management, and patient quality of life.[13–15]

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Over 100 medically relevant metabolites can be quantified by measuring the stoichiometric formation of NAD(P)H in an enzymatic reaction.[16] Nicotinamide adenine dinucleotide (NAD) is an enzyme cofactor central to energy metabolism;[17] its oxidized and reduced forms ($NAD^+$ and NADH) and phosphate forms ($NADP^+$ and NADPH) mediate hundreds of enzyme-catalyzed redox reactions.[18] A variety of methods have been used to measure NAD(P)H for metabolite biosensing,[19] but commercialization of disease-related metabolite biosensors has been limited by poor long-term stability, low specificity, lack of portability, and complex operation. Fluorometric assays are advantageous for metabolite biosensing because of their high sensitivity, rapid visualization, low cost, and high temporal and spatial resolution.[20–22] Semiconductor polymer dots (Pdots) are versatile luminescent nanoparticles that have found widespread use in bioimaging and biosensing[23–32] because they exhibit greater brightness, photostability, and biocompatibility than small dyes and other inorganic fluorescent nanoparticles, allowing greater imaging contrast and longer-term observation.[33–40] However, the application of Pdots to metabolite biosensing remains largely unexplored.

Here we designed a metabolite biosensor that combines an NAD(P)H-sensitive Pdot and a metabolite-specific NAD(P)H-dependent enzyme in a solution- or paper-based assay (Figure 1). Enzyme-catalyzed oxidation of the metabolite generates NAD(P)H; under ultraviolet (UV) illumination, the NAD(P)H quenches the red emission of the Pdot while also fluorescing in the blue region.[41] The NAD(P)H-sensitive Pdot consists of the luminescent conjugated polymer poly[{2-methoxy-5-(2-ethylhexyloxy)-1,4-(1-cyanovinylenephenylene)}-co-{2,5-bis(N,N′-diphenylamino)-1,4-phenylene}] (DPA-CNPPV; structure in the Supporting Information, Figure S1a) and the amphiphilic polymer poly(styrene-co-maleic anhydride) (PSMA). Excitation of the Pdot with UV illumination results in emission by the CNPPV monomer at 627 nm that is quenched by NAD(P)H, along with emission by NAD(P)H at 458 nm. The ratio of emission intensities at 458 nm and 627 nm-or the ratio of blue-to-red-channel emission intensities when using a digital camera and RGB image processing-is used to accurately measure the concentration of the oxidized metabolite. We demonstrate phenylalanine biosensing in human plasma for a phenylketonuria screening test; biosensors for the disease-related metabolites lactate, glucose, glutamate, and β-hydroxybutyrate; and a paper-based assay with smartphone imaging for point-of-care metabolite monitoring.

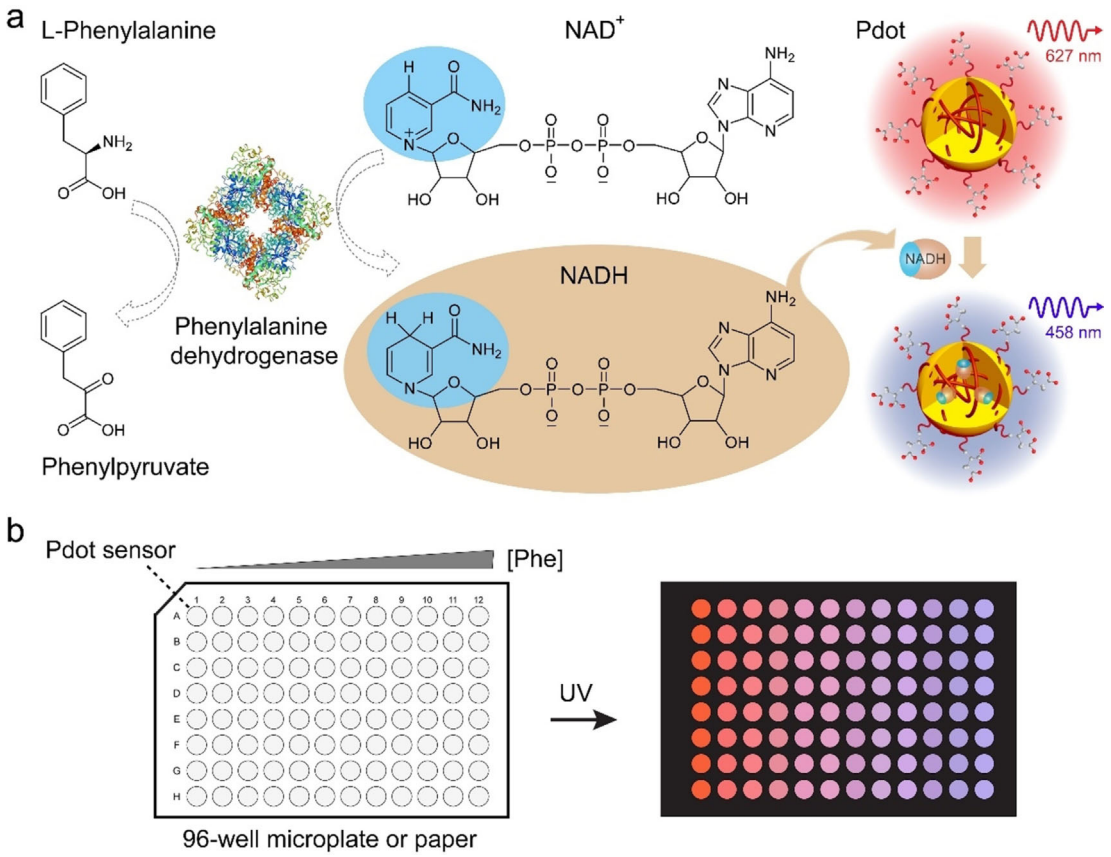


***Figure 1.*** Phenylalanine biosensor for a phenylketonuria screening test. a) Phenylalanine sensing mechanism. Phenylalanine dehydrogenase catalyzes the oxidation of L-phenylalanine by $NAD^+$, resulting in stoichiometric formation of NADH. NADH quenches Pdot fluorescence emission at 627 nm and fluoresces at 458 nm. b) As phenylalanine concentration increases, fluorescence emission upon ultraviolet excitation shifts from red (Pdot emission) to blue (NADH emission). Metabolite concentration is measured ratiometrically-based on the ratio of blue-to-red channel emission intensities, with a digital camera or plate reader-in solution- or paper-based assay formats.

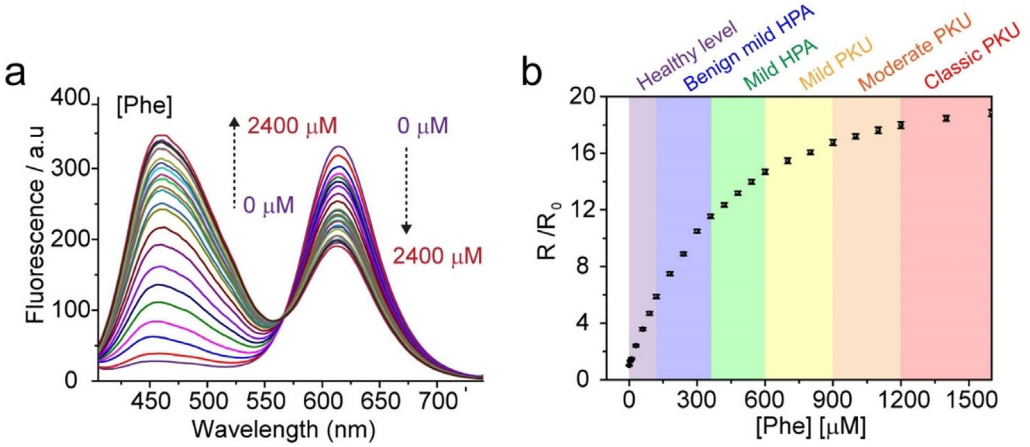


***Figure 2.*** Phenylalanine Pdot biosensor. a) Fluorescence emission of the Pdot biosensor in the presence of phenylalanine dehydrogenase (1 µM) and phenylalanine at 0–2400 µM. b) Ratiometric calibration curve ($R = I_{458nm}/I_{627nm}$; $R_0 = R$ in the absence of phenylalanine ) of the Pdot biosensor as a function of phenylalanine concentration. Colors correspond to different diagnoses. Colors correspond to different diagnoses.

## *Results and Discussion*

An NAD(P)H-sensitive Pdot was prepared by nanoprecipitation of the luminescent polymer DPA-CNPPV and the amphiphilic polymer PSMA using a method developed previously in our lab.[42,43] The Pdot was ca. 19 nm in diameter based on dynamic light scattering, and exhibited a surface zeta-potential of −38 mV (Figure S1b,c). The Pdot has a primary absorption region of 300–400 nm and an emission region of 600–700 nm (Figure S1d). In the presence of NADH

at 2 mM, the emission spectrum changed dramatically, with a large decrease in emission at 627 nm and an increase at 458 nm, the emission maximum of NADH (Figure S2). The Pdot also exhibited reduced fluorescence at 627 nm to NADPH at 2 mM, but showed no response to 2 mM $NAD^+$ or $NADP^+$ (Figure S3).

Phenylketonuria (PKU) is an inherited metabolic disorder that involves reduced metabolism of the essential amino acid phenylalanine due to a defect in phenylalanine hydroxylase.[5] Infants and children with PKU typically develop progressive neurological disease. To create a phenylalanine biosensor, we combined the DPA-CNPPV/PSMA Pdot with phenylalanine dehydrogenase (PheDH), an NADH-dependent enzyme with high specificity for phenylalanine. The biosensor showed an 18.9-fold change in the ratio of emission intensities at 458 nm and 627 nm when titrated with phenylalanine from 0 to 2400 μM, with a limit of detection of 3.5 μM phenylalanine, and a $c_{50}$ of 279.7 μM (Figure 2a,b). The Pdot sensor shows high selectivity against potential interfering metabolites, phenylalanine triggered a large enhancement of emission ratio, while emission-ratio changes by other metabolites were not discernible. This desirable selectivity could be ascribed to the specific catalytic reactions of the enzyme (Figure S4).

Blood phenylalanine levels in PKU patients are managed throughout the patient's life[44,45] because phenylalanine can rise to toxic levels that cause brain damage and other neurological complications. PKU patients are classified according to their serum phenylalanine plasma concentration: 120–360 μM is classified as benign mild hyperphenylalaninemia (HPA), 360–600 μM as mild HPA, 600–900 μM as mild PKU, 900–1200 μM as moderate PKU, and over 1200 μM as classic PKU.[45] The emission ratio ($R/R_0$) showed a linear relationship with phenylalanine concentration within these

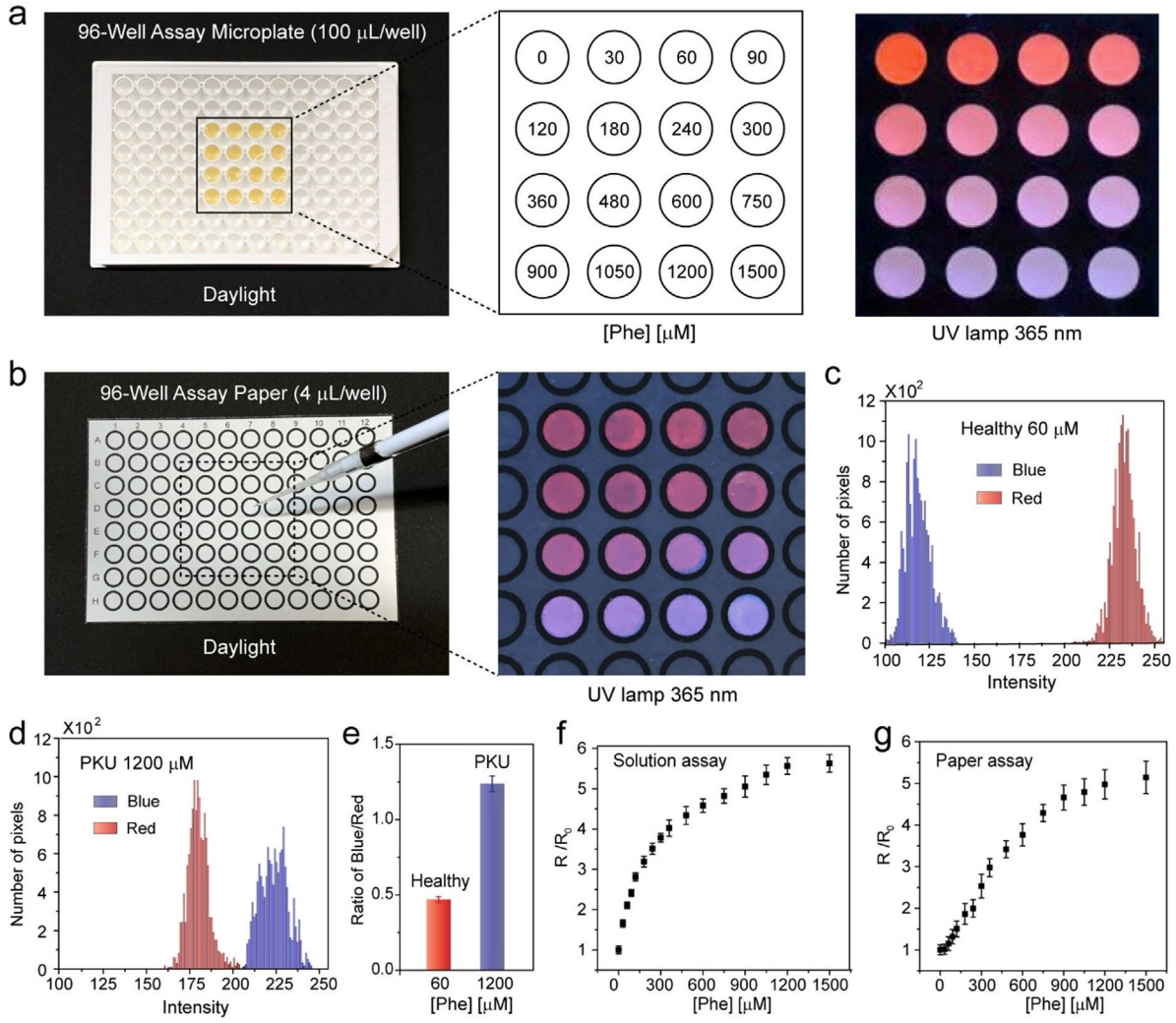


***Figure 3.*** Comparison of paper- and solution-based assays. a) A 96-well plate (left) was loaded with buffer solutions containing the Pdot, phenylalanine dehydrogenase (PheDH) (1 μM), and 0–1500 μM phenylalanine (Phe). The color of emission shifted from red at 0 μM Phe to blue at 1500 μM Phe (right). b) 96-well assay paper was loaded with solutions containing the Pdot, PheDH (2 μM), and 0–1500 μM Phe. c),d) Pixel intensity distributions in a single well in blue and red channels at 60 μM Phe (healthy) and 1200 μM Phe (classic PKU threshold). e) The mean ratio of blue- and re-channel emission increased significantly between 60 μM and 1200 μM Phe. f),g) Ratiometric calibration curve plotting the ratio of blue-channel to red-channel emission intensities ($R$), normalized to the ratio at 0 μM Phe ($R_0$), as a function of Phe concentration for solution- and paper-based assays.

clinical ranges (Figure S5). Table S1 summarizes the performance parameters of the phenylalanine Pdot biosensor including maximal signal change, sensitivity, and resolution in the different clinical ranges.

Methods currently used to measure phenylalanine in PKU patients include the Guthrie bacterial inhibition assay, ferric chloride test, dye-based fluorescence assays, and PCR assays.[46] These methods typically involve complex sample preparation and long test times. We designed a simple paper-based assay that uses a digital camera and requires an incubation period of less than 10 minutes and compared the performance of this assay with that of a similar solution-based assay in a multiwell plate (Figure 3a). The paper assay was prepared by lyophilizing a buffered solution containing the Pdot, $NAD^+$, and PheDH on paper disks. The enzymatic reaction was initiated by adding a small volume of analyte-containing sample onto the test paper (Figure 3b). Images were analyzed with an RGB image-processing algorithm to calculate the average blue- and red-channel intensities within each well from pixel intensity distributions (Figure 3c,d). The ratio of blue- and red-channel intensities was significantly lower at 60 μM phenylalanine (healthy) than at 1200 μM phenylalanine (classic PKU threshold) (Figure 3e). The phenylalanine concentration was calculated from the blue/red emission ratio by using a ratiometric calibration curve obtained by using either a digital camera (Figure 3f,g, Table S2) or a plate reader (Figure S6).

We next evaluated the performance of the paper-based assay when assaying human blood plasma samples. The biosensor was calibrated by analyzing samples in the absence or presence of PheDH (Figure 4a) to correct for interpatient variations in endogenous NADH concentration in blood.[16] We subtracted the value obtained without PheDH from the value obtained with PheDH to obtain the phenylalanine concentration (labeled "difference" in Figure 4b). As a proof of principle for PKU screening applications, we analyzed plasma samples spiked with various concentrations of phenylalanine with the paper assay, and compared the results obtained when using a plate reader (Figure 4c) and a digital camera (Figure 4d) for readout.

Generalization of the assay is important for practical applications of the biosensor. Any metabolite enzymatically oxidized by $NAD^+$ or $NADP^+$ or reduced by NADH or NADPH (Figure S7) can be quantified with this system. We demonstrated ratiometric calibration curves for the disease-related metabolites lactate, glucose, glutamate, and β-hydroxybutyrate using the Pdot together with an NADH-dependent enzyme specific to each metabolite (Figure 5). Lactate detection is important in medical conditions including hemorrhage, respiratory failure, hepatic disease, and sepsis; glucose monitoring is important in managing diabetes; glutamate monitoring is useful in diagnosis and monitoring of neurodegenerative diseases; and β-hydroxybutyrate sensing is used to detect hyperketonemia. Over 100 medically-relevant metabolites are potentially compatible with this system for quantification as listed in Table S5.

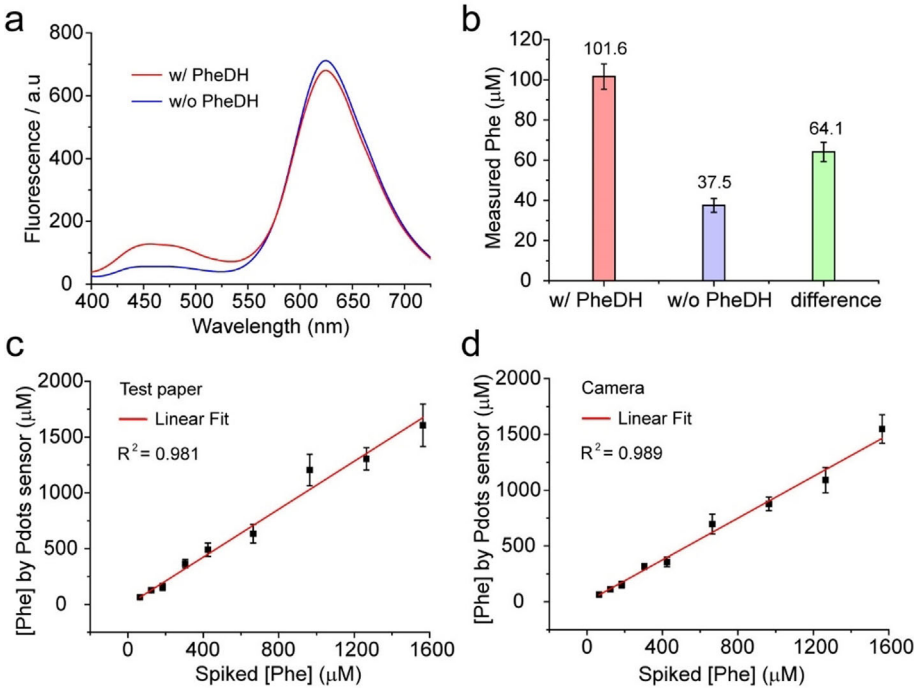


***Figure 4.*** Measurement of phenylalanine in human blood plasma. a) Fluorescence emission of the phenylalanine biosensor in the presence or absence of phenylalanine dehydrogenase (PheDH). b) Background correction for endogenous NADH levels. The sensor without PheDH measures endogenous NADH; subtracting this value from the value with PheDH yields the phenylalanine concentration. Phenylalanine spiked into plasma samples was measured using c) a fluorescence plate reader and (d) a digital camera for readout.

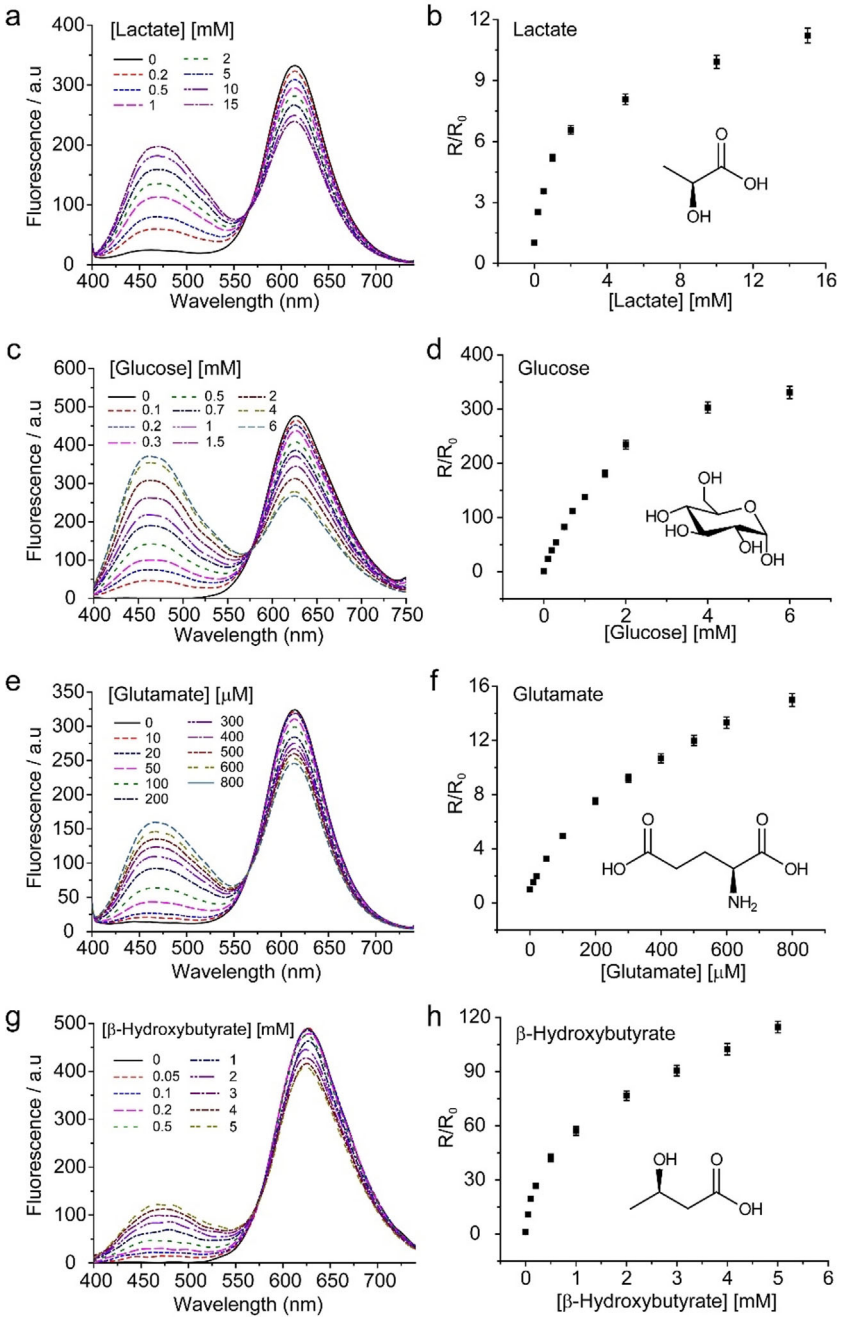


***Figure 5.*** Generalizable metabolite Pdot biosensor. a) Fluorescence emission spectra of the sensor with lactic dehydrogenase in the presence of various concentrations of lactate, and b) corresponding ratiometric calibration curve. c) Fluorescence emission spectra of the sensor with glucose dehydrogenase in the presence of various concentrations of glucose, and d) corresponding ratiometric calibration curve. e) Fluorescence emission spectra of the sensor with glutamic dehydrogenase in the presence of various concentrations of glutamate, and f) corresponding ratiometric calibration curve. g) Fluorescence emission spectra of the sensor with β-hydroxybutyrate dehydrogenase in the presence of various concentrations of β-hydroxybutyrate, and h) corresponding ratiometric calibration curve.

## Conclusion

We developed a ratiometric metabolite biosensor that integrates an NAD(P)H-sensitive Pdot and a metabolite-specific NAD(P)H-dependent enzyme for use in metabolic assays at the point of care. The analyte is specifically oxidized in an NAD(P)H-dependent enzyme-catalyzed reaction, and the sensor emission shifts from red to blue at elevated NAD(P)H concentrations, allowing accurate ratiometric measurement of metabolite concentration. This Pdot-based metabolite assay has broad potential utility for point-of-care testing to improve disease diagnosis and management.

## Conflict of Interest

J.Y. and D.T.C. have a financial interest in Lamprogen, which has licensed the described technology from the University of Washington.

**Keywords:** metabolite biosensors · nicotinamide adenine dinucleotide · paper assays · point of care · semiconducting polymer dots

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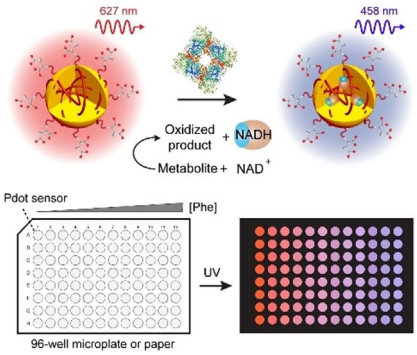
# Research Articles

**Biosensors**

H. Chen, J. Yu, J. Zhang, K. Sun, Z. Ding, Y. Jiang, Q. Hu, C. Wu, D. T. Chiu* ——— ▮▮▮▮–▮▮▮▮

Monitoring Metabolites Using an NAD(P)H-sensitive Polymer Dot and a Metabolite-Specific Enzyme




A metabolite biosensor is developed by integrating an NAD(P)H-sensitive semiconducting polymer dot and a metabolite-specific NAD(P)H-dependent enzyme. Metabolite concentration can be measured ratiometrically with high sensitivity and specificity. Phenylalanine biosensing in human plasma is shown for a phenylketonuria screening test, quantitation of other disease-related metabolites, and a paper-based assay with smartphone imaging.