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Enhanced Chemiluminescence of a Superior Luminol Derivative Provides Sensitive Smartphone-Based Point-of-Care Testing with Enzymatic μPAD

Simone Rink,[a] Axel Duerkop,[a] and Antje J. Baeumner*[a]

Chemiluminescence (CL) provides ideal conditions for point-of-care testing (POCT) with wide dynamic ranges, superior sensitivities, and detection simplicity. It has not arrived routinely in the POCT field due to naturally low quantum yields of typical probes and the lack of sensitive low-cost detection devices. Here, we developed a universal microfluidic paper-based analytical device (μPAD) using L-lactate as model analyte. We demonstrate that a smartphone camera can compete with a scientific CCD camera as performance benchmark when using the strong CL emitter, *m*-carboxy luminol, resulting in extraordinary signal-to-noise ratios of 67. The μPAD provides CV < 10%, stability at room temperature for ≥ 3 months and simple processing. Furthermore, the μPAD enables the detection of picomoles of the luminophore providing additional design flexibility. Thus, this new CL-μPAD is available for translating the many CL standard analytical assays performed in **microtiter plates**, microarrays or other more complex detection strategies to the POC.

Introduction

One of today's challenges in analytical research is the development of highly sensitive and selective analysis platforms, which can easily be carried out in-field by trained and non-trained personnel. For this, sensitive and low-cost assay principles are required, which are easy to operate.[1] Chemiluminescence (CL) provides simple, portable and cost-efficient detection due to its independency of external light sources and may use detectors as easy and popular as smartphone cameras.[2] Yet, it competes with regard to detection limits with radioanalytical assays[3] and allows ultrasensitive measurements down to zeptomole detection limits due to its background-free nature.[4] It further convinces with short measuring times, wide dynamic ranges and universal (bio)analytical application, such as in routine clinical laboratories for immunoassays or DNA probe assays, as well as in biochemical research for reporter gene assays, cell viability assays and many more.[3] The Whitesides group on the other hand pioneered in a new class of point-of-care testing (POCT) platforms, the microfluidic paper-based analytical devices (μPADs).[5] Combining CL detection with this new class of POCT, which shows already promising prospects for mobile POCT with broad diagnostic applications,[6] allows for ultrasensitive but still affordable point-of-care (POC) platforms. μPADs combine the flexibility of microfluidic total analysis systems (μTAS) with regard to the integration of multiple processes, multiplexing and variability in analytical assays with the simplicity and cost-efficiency of lateral flow assays (LFA).[7] However, the majority of current paper-based microfluidic systems are dominated by colorimetric, fluorescent or electrochemical detection.[8,9] The drawbacks of these detection techniques, are either their lack in sensitivity, challenging manufacturing or their requirement of costly detection devices.[10] Thus, current μPADs struggle, among others, with reproducibility, modest sensitivity, insufficient specificity[8] and the lack of detection devices with freely available evaluation software.[11] Especially colorimetric μPADs often suffer from inhomogeneous color development[12] e.g., caused by bleaching of colored products over time or their overoxidation due to ongoing enzymatic reactions, ambient light imbalance, and shadow formation while image taking and variation in color generation algorithms of different camera manufacturers[13] hampering commercialization.[14] CL, on the contrary, relies on the generated light intensity which can be captured without external illumination and extracted from the unprocessed raw monochromatic images and is thus independent of the generated color signal, shooting angle and external illumination. Despite CL's great potential as detection technique, it has not yet arrived routinely in the POCT field. This is probably due to the transient signal and in general low quantum yields (1% for CL reactions) and thus low degrees of emitted light of typical CL reagents.[15] Furthermore, the lack of sensitive low-light sensor chips for non-scientific and affordable cameras limited the progression as well. Thus, ideally, expensive cooled charge-coupled device (CCD) cameras or photomultiplier tubes[15,16] are required for sensitive detection which do not fit the budget nor the application mode of POCT. Together with easier readout strategies, minimal instrumental requirements

[a] *S. Rink, Prof. Dr. A. Duerkop, Prof. Dr. A. J. Baeumner*
Institute of Analytical Chemistry, Chemo- and Biosensors
University of Regensburg
Universitätsstraße 31
93053 Regensburg (Germany)
E-mail: antje.baeumner@ur.de

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along with the sensor chip improvement of smartphone cameras toward higher light sensitivity (the ISOCELL image sensor from Samsung, the SuperSpectrum Sensor from Huawei, the ExmorRS from Sony or the OmniVision image sensors), CL poses a powerful and elegant alternative detection principle.[15] In addition, with the enhanced CL signal of the herein studied *m*-carboxy luminol, CL is now ready for inexpensive sensitive POCT solutions.

We show in this study a universal μPAD principle for the detection of typical biomarkers based on enzymatic conversion with the byproduct H_2O_2 such as the reaction of L-lactate into pyruvate through lactate oxidase (LOx). Especially L-lactate has recently raised interest as it is not only promising as POCT solution for clinical practice and emergency care, but relevant as well for the ongoing trend of self-monitoring of fitness levels with easy, portable and economic analytical devices.[17,18] Depending on the biological fluid, lactate concentrations can range from 0.1 mmol L^{-1} to 2.5 mmol L^{-1} in saliva[19] and can also be as high as 115.8 mmol L^{-1} in sweat after exhaustive exercise.[20] Thus, detection over a vast dynamic range is required to cover the complete relevant area with high resolution. This is provided by CL in general, as it shows low background signals and broad linearity and is furthermore strengthened by the increased CL emission of *m*-carboxy luminol used in this work. Furthermore, we studied the detection of the luminophore itself in a generalized paper-based microfluidic approach and achieved higher signal-to-noise ratios along with lower limits of detection. This highlights our CL probe not only as substrate but also as chemiluminescent label for biomolecules which introduces extra selectivity to CL-μPADs and overall broader application possibilities.

Results and Discussion

The obtained extraordinary chemiluminescence enhancement of *m*-carboxy luminol over standard luminol in microtiter plate-based bioassays[21] encouraged us to transfer our findings to a microfluidic paper-based analytical device (μPAD) to investigate its feasibility toward a sensitive POCT system. Here, the determined photophysical characteristics of *m*-carboxy luminol in Rink et. al. laid the foundation for this work. The initial design of the μPAD (Figure 4A) consists of two individual zones, one sample and four detection zones which are connected through paper-based channels allowing to record the entire CL reaction. The sample zone was either modified with lactate oxidase (LOx) or simply used as bare application zone. The four detection zones contain drop-coated and catalyst and allow multiple determination within one paper substrate with good reproducibility and a coefficient of variation (CV) $< 10\%$. Similar results were obtained when using three individual paper substrates, independent from the employed analyte. Here the CV is again mainly $< 10\%$ in both designs (Figure S1, Figure S2) which is remarkable considering the manual fabrication process and the heterogenous nature of the paper. Furthermore, the design allows to extract the entire CL signal due to the designed channel structure and the accompanied reaction delay. The channel structure additionally provides flexibility to introduce structural features such as incubation areas or waste zones if needed. The original design was used for direct analyte detection when no prior conversion of the sample and incubation, respectively, was needed. We investigated three major catalysts, namely cobalt, hemin and horseradish peroxidase (HRP)[21] regarding their catalytic and analytic performance. We first tested our system with the initial design and H_2O_2 as analyte to identify ideal conditions before switching to the enzymatic μPAD for the analysis of L-lactate in synthetic sweat. As a second approach we altered the assay strategy toward the luminophore as analyte which suggests that *m*-carboxy luminol has indeed a great potential as ultrasensitive chemiluminescence label in POC devices.

Establishing chemiluminescence reaction on simple μPAD for H_2O_2 detection

Detection of H_2O_2 has great relevance in bioanalysis. Besides its role as signaling messenger and biomarker for oxidative stress, it functions as substrate for peroxidases and is often a byproduct of oxidase-based enzymatic reactions. The latter can be exploited for the detection of clinically relevant metabolites such as glucose, lactate or uric acid.[22] Thus, we preliminary studied our chemiluminescence μPADs for H_2O_2 detection to design the initial μPAD platform. We observed enhancement in sensitivity (up to three times) over standard luminol and were able to detect H_2O_2 down to low μmolar concentrations (Table S1) which is sufficient for most of these metabolites. All three tested catalyst systems showed an increase in signal response with *m*-carboxy luminol. When using hemin and HRP as catalyst, we obtained a linear relation between 0 to 2 mmol L^{-1} H_2O_2 (Figure 1), whereas with cobalt the CL intensity increased non-linearly in the chosen concentration range.

This correlates very well with our observation in the microtiter plate,[21] where the CL reaction with cobalt exhibits non-linear behavior already starting at approximately 1 mmol L^{-1} H_2O_2, which makes cobalt a less suitable catalyst for a simple POC application. We decided to use hemin as catalyst due to its similar performance to HRP (see SI, Table S2). Whereas standard colorimetric μPADs for metabolites such as uric acid, glucose and L-lactate rely on HRP for color generation, the μPAD developed herein has the unique feature to function without the additional peroxidases, simplifying the final preparation, recording and storage conditions. Although slightly lower LODs were obtained when employing *m*-carboxy luminol, the more decisive advantage here is the significant improvement in resolution by an up to three times steeper slope.

Combined with a signal-to-noise ratio of around 20 for *m*-carboxy luminol in contrast to around eight for luminol, this new luminol is an ideal label for highly sensitive but low-cost POCT systems (Figure 1). To test our μPAD system for real applications, we transferred these findings toward an enzymatic μPAD using LOx to detect L-lactate through the oxidation-product H_2O_2. The μPAD contains all reagents in a

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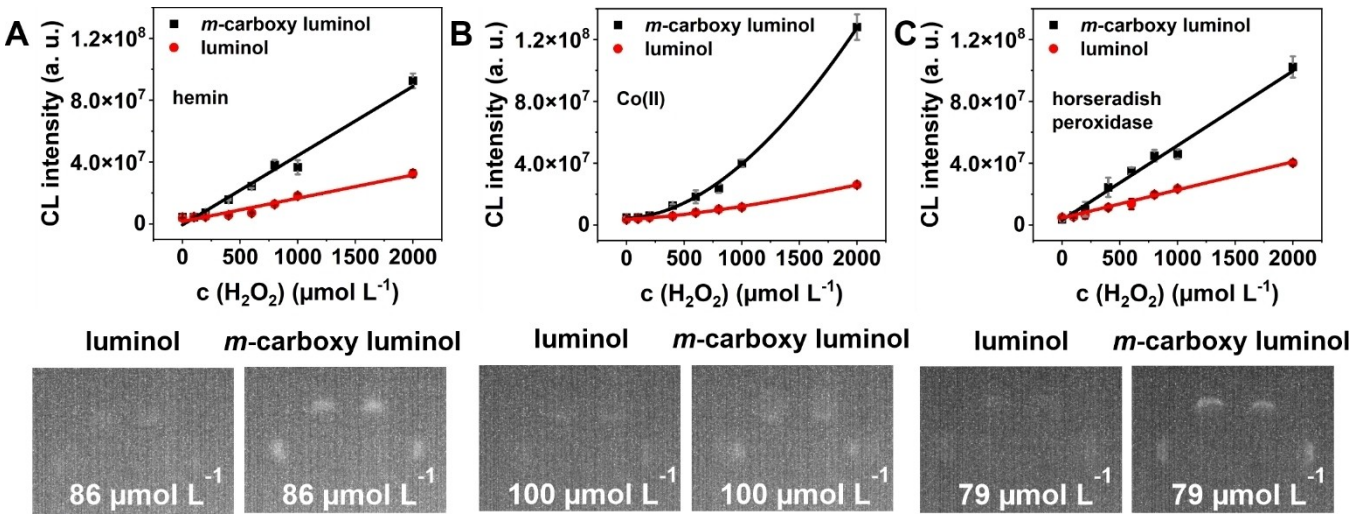

Figure 1. Dose-response curve for H_2O_2 detection with (A) hemin, (B) Co(II) and (C) horseradish peroxidase (HRP) including validation of calculated LOD, with paper substrate for *m*-carboxy luminol (black) and luminol (red) with 1 nmol luminophore, 1 nmol Co(II) or hemin and 0.3 U HRP with 1 nmol *p*-coumaric acid were dried in the detection zone, 30 μL H_2O_2 in 0.1 mol L^{-1} carbonate buffer (pH 10.5) were applied to the sample zone, linear fitting was performed by Origin2020 with $R^2 = 0.978$ (black) and $R^2 = 0.954$ (red) for hemin and $R^2 = 0.984$ (black) and $R^2 = 0.994$ (red) for HRP and four-parameter logistic fit was performed by Origin2020 with $R^2 = 0.978$ (black) and $R^2 = 0.998$ (red) for Co(II), mean ± SD (n = 4), 15 images, 2 s exposure, adjustment of brightness and contrast for each luminophore pair image for better visualization.

dried state. When L-lactate is added to the sample zone, it is oxidized by LOx to pyruvate generating a stoichiometric amount of H_2O_2 which reacts subsequently with the luminophore to generate light (Figure 2A).

Advanced μPAD design enables enzymatic reaction and detection optimization

To obtain a homogenous signal when employing LOx to oxidize L-lactate, an incubation step prior to the CL reaction is needed to allow for efficient conversion. Hence, refining our original μPAD design for the H_2O_2 detection with a wax barrier upstream of the sample zone which can be bridged with a functionalized filter paper strip, allowed efficient sample conversion (Figure 4B). We tested different drying times and concentrations of LOx along with different substrate incubation times to determine ideal execution conditions (Figure S3). Incubation for 10 min with 5 U μL^{-1} LOx prior to detection was finally chosen (see SI). In a second line of research, we tested ideal recording conditions to maximize signal collection. Multiple images were taken over a specific time span and subsequently merged before analysis (see SI). To account for time delays and to balance noise generation, 15 images for the CCD camera and nine images for the smartphone camera were recorded (Figure S4). Furthermore, we are currently working on a software-based solution which filters the accumulated noise to improve the overall signal-to-noise ratio especially after merging. Ultimately, we can conclude that using a scientific CCD camera *versus* a simple smartphone camera further demonstrates the benefit of a stronger emitting detection probe when switching from lab to in-field equipment as a strong response allows to use less sensitive detection devices (e.g. cell phones or photodiodes) while maintaining the overall performance.

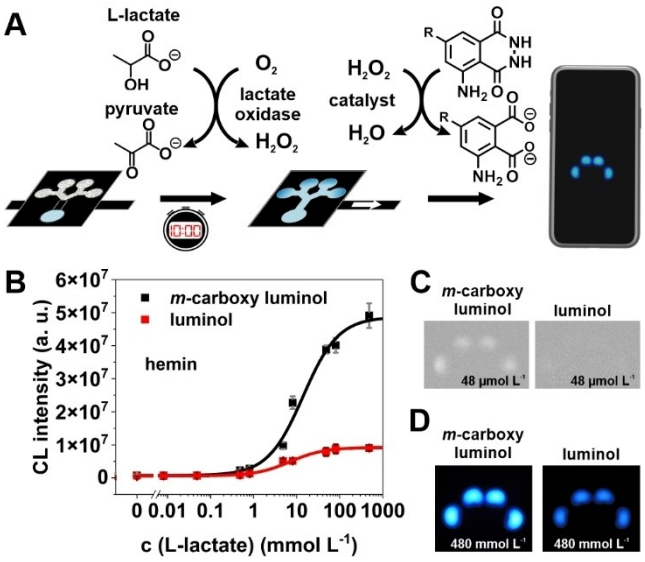

Figure 2. Chemiluminescence L-lactate determination with smartphone (A) illustration of workflow (B) calibration curve of L-lactate in synthetic sweat matrix, using μPADs with stacked 16-bit raw images mean ± SD (n = 4), (C) stacked raw images close to LOD with both luminophores (D) single RGB images for both luminophores at maximum intensity.

Benchmarking analytical performance of advanced μPAD toward L-lactate with a standard smartphone camera against a scientific CCD camera

The CL recording was done in a simple dark box with the distance adjusted to the respective front focal distance of both cameras (smartphone camera: 8 cm, CDD: 47.8 cm). Transitioning the assay to smartphone recording the assay remained the same while the recording process was optimized (see SI, chapter 4). While the smartphone camera provided lower signal responses, its signal-to-noise ratio (S/N) of 67 was in fact superior to that of the CCD camera (S/N of 38) in the enzymatic μPAD. Whereas full conversion of L-Lactate is anticipated in absolute determinations, only partial, but still quantifiable conversion into H_2O_2 is observed in most biosensors and intended in our μPAD (Figure S2). This enables a dynamic range expansion of our analysis platform while avoiding detector saturation or early substrate inhibition. However, due to the incomplete conversion of L-lactate the generated signal is not as intense as for the equivalent H_2O_2 sample (Figure S2). Our more intense CL probe (Figure 2D, single images of maximum intensity at 480 mmol L^{-1} L-lactate) compensates for this allowing even higher resolution despite lower product concentration in contrast to standard luminol. Despite this, the calculated limits of detection (LOD) for luminol and *m*-carboxy luminol (Figure 2B) are similar ($LOD_{m\text{-carboxy luminol}}$: 0.03 mmol L^{-1}, $LOD_{luminol}$: 0.02 mmol L^{-1}), however, when actually measuring those, only the *m*-carboxy luminol exhibits a visible CL signal (Figure 2C). In any event, with the obtained LODs the clinically relevant range of 0.5–2 mmol L^{-1} as baseline serum concentration is easily covered, similar to values >2 mmol L^{-1} which are typically associated with hyperlactatemia.[23] Presupposing a correlation between blood and sweat lactate, our μPAD allows for simple non-invasive lactate detection over a fast dynamic range (0.03–100 mmol L^{-1}) in a matrix with low potential of interfering species (99% water, electrolytes, metabolites and micronutrients).[18] However, the positive correlation between blood and sweat lactate is still under vivid debate in the field and non-controversial scientific data have not yet been provided.[18] Even extreme L-lactate levels up to 100 mmol L^{-1} which are possible after exhaustive exercises are quantifiable with a higher resolution when using *m*-carboxy luminol over luminol. Similar results were obtained with the CCD camera (Figure S5). The slightly higher LODs ($LOD_{m\text{-carboxy luminol}}$: 0.09 mmol L^{-1}, $LOD_{luminol}$: 0.3 mmol L^{-1}) are most likely caused by the increased background noise of the CCD camera (Figure S5C, Table S3) and hence the decreased S/N ratio.

The unique combination of luminophore, μPADs and smartphone enable a point of care lactate detection with highest sensitivity and a broad dynamic range. For example, Li et al. obtained a similar LOD as with our smartphone approach. However, they employed an ultraweak luminescence analyzer to obtain such sensitivity values and report a dynamic range of only 0.02–5 mmol L^{-1}.[24] Roda et al., on the other hand, developed a nitrocellulose-based cartridge system being a μPAD in a much broader sense with a LOD of 0.1 mmol L^{-1} for lactate in sweat which further needs special smartphone-dependent detection accessories.[25] Li et al., introduced a colorimetric μPAD with smartphone detection for inter alia lactate with an LOD of 0.03 mmol L^{-1} and a linear range from 0.04–24 mmol L^{-1}, but simultaneously show the color alteration with increasing concentrations within their assay when using color reactions.[26] Furthermore, the related electrochemiluminescence (ECL) approach for the enzymatic detection of biomarkers on paper substrate showed promising results toward improved sensitivities.[27] Yet, despite its advantage of controlled reaction initiation, the ECL approach requires to apply a certain voltage to the paper device. The additional instrumental requirements may diverge from the overall POCT idea in their current state but may compete with paper-based CL in the future using smart device fabrication to avoid manual cable connections. Furthermore, the stability of the drop coated enzyme on the electrode is yet to be determined. A true improvement with our new CL probe regarding the sensitivity was obtained when changing from the detection of H_2O_2 to the luminophore itself with low picomole detection limits (Figure S6, Table S4). This is especially evident when considering the LOQ which is the diagnostically more relevant value.

Application to real samples

To evaluate the applicability and accuracy of the developed μPAD to real samples, we detected L-lactate in synthetic sweat. The synthetic sweat was prepared according to DIN 53160-2. It contains a defined amount of lactic acid and provides a representative sample matrix which is common in literature.[28,29] As lactic acid consists of D-lactate and L-lactate, the L-lactate level was first determined with a commercial colorimetric microtiter plate assay to be (4.5 ± 0.2) mmol L^{-1}.[21] With both luminophores, the determined concentration correlates very well with the value obtained by the commercial assay (Table 1). Considering the intensity values the coefficient of variation (CV) is still around 10% (Table S5). With error propagation this value rises to ≤ 18%. We believe that this can

Table 1. Validation of developed chemiluminescence μPAD for L-lactate by determining L-lactate in synthetic sweat (DIN 53160-2)[28,29] with a commercial assay.

Lactate assay kit	Luminophore	L-lactate in synthetic sweat (DIN 53160-2)
custom-made smartphone (within this study)	*m*-carboxy luminol	5.2 ± 0.9 (CV: 18%)[a]
	luminol	3.5 ± 0.3 (CV: 10%)[a]
custom-made CCD (within this study)	*m*-carboxy luminol	4.5 ± 0.5 (CV: 11%)[a]
	luminol	3.23 ± 0.06 (CV: 2.0%)[a]
commercial (Sigma Aldrich)	–	4.5 ± 0.2 (CV: 4.2%)[b]

A Tukey test at the 0.05 significance level was performed and no significant difference of the mean of the commercial assay to the chemiluminescence μPADs with *m*-carboxy luminol was obtained. [a] mean $\pm$ SD (n = 4), [b][21], mean $\pm$ SD (n = 3).

be easily reduced by reducing the manual preparation steps, such as the pretreatment of the detection zone.

No significant difference for *m*-carboxy luminol was obtained between the L-lactate levels with the CCD camera or the smartphone camera toward the commercial assay, making both detectors valuable choices. Although only the CCD approach yields a significant statistical difference between the obtained L-lactate level toward the commercial approach, luminol tends to yield slightly underestimated results in the smartphone approach as well, pointing again toward *m*-carboxy luminol as superior probe. Finally, when diluting the synthetic sweat sample in synthetic sweat matrix, the CL response decreases linearly with an R^2 of 0.996 (Figure S7) excluding matrix interference on the enzymatic and CL reaction. This is also shown in Figure S2 where L-lactate was measured in ultrapure water and synthetic sweat without any significant effect on the generated CL signal.

Stability of developed μPAD for L-lactate

In view of a costumer-orientated solution, the stability of the employed reagents is of high relevance and their activity needs to be maintained throughout long-time storage. Especially, enzymes pose a critical reagent as they are pH-sensitive and can be affected by varying environmental conditions such as temperature, humidity or exposure to proteases.[30] However, it was shown that μPADs with physisorbed enzymes can retain their activity for up to 10 weeks when stored in a sealed container at 4 °C.[31] Yet, these storage conditions are not always feasible. The stability of the μPAD developed herein was thus tested toward its robustness against elevated temperature, and enzyme batch-to-batch variation. The developed μPAD was weekly tested with a standardized 20 mmol L^{-1} L-lactate solution and enzyme functionality was maintained for three months when stored at room temperature dried on the μPAD and in solution at 4 °C and applied before use without significant activity loss and no batch-to-batch variation of the applied enzyme (Figure S8B). Together with the stable response from the dried luminophore on paper which was routinely tested with 1 mmol L^{-1} H_2O_2 (Figure S8A), a robust μPAD was developed which can be stored at room temperature for at least three months.

Conclusion

In this proof-of-principle study, we investigated different strategies for chemiluminescence μPADs to expand the current POCT portfolio. We propose that exchanging the dominant colorimetric, simple readout strategy, with an equally simple approach using chemiluminescence, will address exactly the challenge of limited sensitivity, that μPADs still endure. This enables μPADs to provide simple solutions to analytical challenges, such as the performance of multiplex reactions and the easy expansion of the assay layout into the 3D space. We demonstrated that with a stronger emitting luminol a ubiquitous detector i.e., a normal smartphone camera, can maintain the simplicity of detection strategy while providing superior detection capabilities. Our developed μPAD system for L-lactate further could facilitate non-invasive and fast sample collection due to the inherent material characteristics of the paper-based device, simplifying and speeding up the overall process. Moreover, this enzymatic μPAD can be stored at room temperature for at least three months enabling simple in-field application. This concept can easily be expanded to other enzymatic reactions with H_2O_2 as side product and, together with the incubation barrier, sufficient conversion can be easily accomplished. Furthermore, in contrast to colorimetric μPADs, a wide dynamic range is covered without the alteration of the signal due to overoxidation and thus color change. In comparison to other CL POCT strategies, our approach convinces by its simple procedure already directed toward user-friendliness. In contrary to others needing sophisticated detectors that are not fit for POCT to reach low limits of detection, here a simple smartphone camera reached similar sensitivity levels due to the strong CL emitter used. Finetuning of the measurement setup is still possible by developing a smartphone adapter integrating macro lenses to correct for the optics[32] which would increase light collection and guarantee the exclusion of ambient light but was not the main focus in this study. Additionally, a tailored software addressing user-friendly evaluation and compression of background signals is currently under evaluation to further simplify the process and the accompanied user-interventions. This would provide a low-cost μPAD system with excellent analytical performance ready for the POC.

Experimental Section

Chemicals and consumables. All chemicals were commercial HPLC grade and were used without purification. Standard chemicals were purchased from Merck. Horseradish peroxidase (HRP) was purchased from Merck (Darmstadt, Germany) and a 300 U mL^{-1} stock solution in 1×PBS buffer, pH 7.4 was prepared. Lactate oxidase (LOx) was obtained from AG Scientific (San Diego, USA) and a 100 U mL^{-1} in 1×PBS buffer, pH 7.4 was prepared. Both enzymes were aliquoted and stored at 4 °C and respective working solutions were prepared freshly before each measurement. *m*-Carboxy luminol was custom-made by Taros Chemicals GmbH & Co. KG (Germany), according to a standard procedure.[33] Synthetic sweat according to DIN 53160-2 was purchased from Synthetic Urine e. K. company (Nußdorf, Germany). For all experiments ultrapure water was used and stock solutions were prepared for luminol (1 mmol L^{-1} in 0.1 mol L^{-1} carbonate buffer, pH 10.5), *m*-carboxy luminol (1 mmol L^{-1} in 0.1 mol L^{-1} carbonate buffer, pH 10.5), hemin (1 mmol L^{-1} in 0.1 mol L^{-1} carbonate buffer, pH 10.5), Co(II) (1 mmol L^{-1} in ultrapure water), *p*-coumaric acid (1 mmol L^{-1} in ethanol, 96 %) and L-lactate (10 mmol L^{-1}). For H_2O_2 the stock solution (100 mmol L^{-1} in ultrapure water) was freshly prepared before each measurement.

Fabrication of paper substrate. The fluidic layout was designed by using CorelDraw 2017 and printed with a Xerox ColorQube™ 8580 wax printer, on chromatography paper Grade 1 CHR (3001-917, GE Healthcare Life Science, Germany). The paper (Figure 3B) was cut to the respective size and the wax was melted on a heating plate for approximately 30 s at 200 °C. After the melting process the back of

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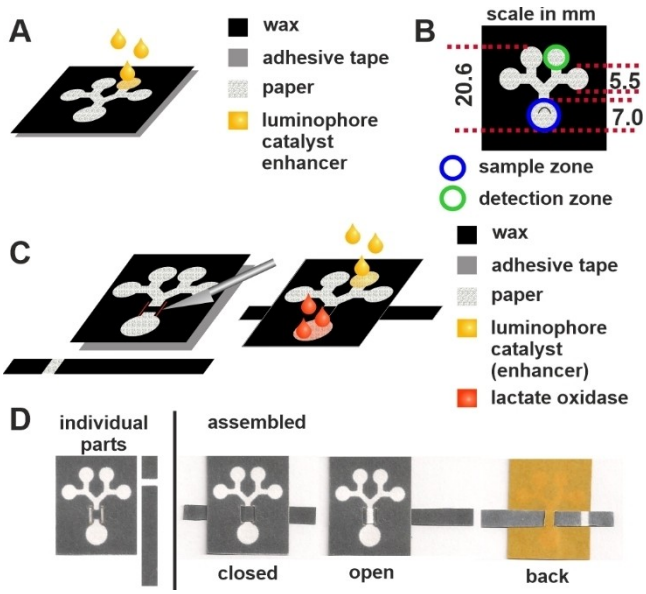

Figure 3. Fabrication of the paper substrate with (A) layout for H_2O_2 detection and luminophore detection, (B) dimensions of the paper substrate, (C) layout for the L-lactate assay and (D) real images of the μPAD.

the paper substrate was sealed with adhesive tape (927, 3M™). The final paper substrate was produced by drop-coating of each 1 μL luminophore, catalyst and enhancer (only for HRP) on the detection zone (Figure 3A). The solutions were allowed to dry in between. The paper substrate was ready to use after all reagents were dried on the filter paper. For lactate determination the paper substrate was adjusted with an additional wax barrier including a bridging strip partially modified with wax (Figure 3C, D).

Measurement on paper substrate. The paper device was placed in a dark box with a Lumenera LW135RM CCD camera and 30 μL of analyte solution were applied to the sample zone. After the analyte solution reached the capturing start point (Figure 4A(2)) the box was closed and 15 images with 2 s exposure time in stacking mode were recorded. For luminophore detection the detection zones were prepared with varying luminophore concentrations. For H_2O_2 detection, varying HP concentrations were applied to the sample zone (Figure 4A). For L-lactate determination, 4 μL of LOx (5 U μL^{-1}) were dried on the sample zone before L-lactate in synthetic sweat matrix was added. L-lactate was incubated for 10 min on the sample zone before the barrier was removed (Figure 4B). For the CCD camera 15 images with 2 s exposure in stacking mode were recorded after the solution reached the capturing start point. For detection with a Samsung S21 5G smartphone, an image series of 9 images was recorded manually with 30 s exposure time and ISO 800. The images were taken each minute after the solution reached the detection zone and stacked afterwards through ImageJ before post processing. CL intensity was determined with ImageJ from the stacked image. The limit of detection (LOD) and the limit of quantification (LOQ) were determined according to $y_{LOD} = A1 + 3 \times \sigma_{blank}$ and $y_{LOQ} = A1 + 10 \times \sigma_{blank}$, respectively, if a four-parameter logistic fit was applied. For linear fitting, the LOD and LOQ were calculated according to $x_{LOD} = 3 \times \sigma_{blank}$/slope and $x_{LOQ} = 10 \times \sigma_{blank}$/slope.

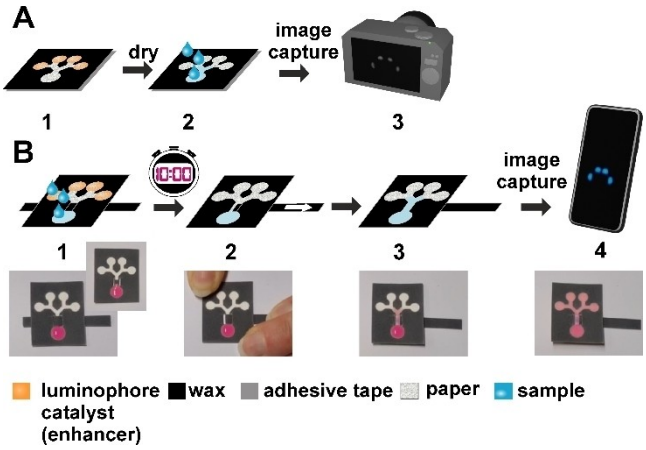

Figure 4. Stepwise illustration of the assay procedure in (A) for H_2O_2 detection and luminophore detection with (1) applying sample to sample zone, (2) signal development and (3) taking images with a CCD camera and in (B) for the L-lactate assay with (1) applying sample to sample zone, incubation for 10 min, (2) opening sample chamber (3) signal development and (4) taking images with either a smartphone or CCD camera, schematic illustration, and real images with sulforhodamine B dye solution for better illustration.

All measurements were conducted at 22 °C and 38 % humidity. A circular region of interest (ROI) was used covering the area of the detection zone and the mean gray value was determined by ImageJ (Fiji, 2.0.0-rc-67/1.52c).[34] The mean gray values were multiplied by the evaluation area to yield the final CL signal response. For the enzymatic μPAD, the S/N ratio was determined as relative measure, using the top asymptote (A2) as signal and bottom asymptote (A1) of the calibration curve as noise.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords: enzymes · luminescence · paper-based microfluidics · point-of-care testing · sweat analysis

[1] N. Jiang, N. D. Tansukawat, L. Gonzalez-Macia, H. C. Ates, C. Dincer, F. Güder, S. Tasoglu, A. K. Yetisen, *ACS Sens.* **2021**, *6*, 2108.
[2] M. Zangheri, L. Cevenini, L. Anfossi, C. Baggiani, P. Simoni, F. Di Nardo, A. Roda, *Biosens. Bioelectron.* **2015**, *64*, 63.
[3] L. J. Kricka, *Anal. Chim. Acta* **2003**, *500*, 279.
[4] W. R. G. Baeyens, S. G. Schulman, A. C. Calokerinos, Y. Zhao, A. M. García Campaña, K. Nakashima, D. de Keukeleire, *J. Pharm. Biomed. Anal.* **1998**, *17*, 941.
[5] A. W. Martinez, S. T. Phillips, G. M. Whitesides, E. Carrilho, *Anal. Chem.* **2010**, *82*, 3.

[6] M. M. Gong, D. Sinton, *Chem. Rev. (Washington, DC, U. S.)* **2017**, *117*, 8447.
[7] a) S. Shrivastava, T. Q. Trung, N.-E. Lee, *Chem. Soc. Rev.* **2020**, *49*, 1812; b) A. M. López-Marzo, A. Merkoçi, *Lab Chip* **2016**, *16*, 3150.
[8] C. Carrell, A. Kava, M. Nguyen, R. Menger, Z. Munshi, Z. Call, M. Nussbaum, C. Henry, *Microelectron. Eng.* **2019**, *206*, 45.
[9] a) L.-M. Fu, Y.-N. Wang, *TrAC Trends Anal. Chem.* **2018**, *107*, 196; b) A. Nilghaz, L. Guan, W. Tan, W. Shen, *ACS Sens.* **2016**, *1*, 1382.
[10] W. Zheng, K. Wang, H. Xu, C. Zheng, B. Cao, Q. Qin, Q. Jin, D. Cui, *Anal. Bioanal. Chem.* **2021**, *413*, 2429.
[11] B. R. Sun, A. G. Zhou, X. Li, H.-Z. Yu, *ACS Sens.* **2021**, *6*, 1731.
[12] H. Jang, J.-H. Park, J. Oh, K. Kim, M.-G. Kim, *ACS Sens.* **2019**, *4*, 1103.
[13] G. G. Morbioli, T. Mazzu-Nascimento, A. M. Stockton, E. Carrilho, *Anal. Chim. Acta* **2017**, *970*, 1.
[14] P. Wang, L. J. Kricka, *Clin. Chem. (Washington, DC, U. S.)* **2018**, *64*, 1439.
[15] A. Roda, M. Mirasoli, E. Michelini, M. Di Fusco, M. Zangheri, L. Cevenini, B. Roda, P. Simoni, *Biosens. Bioelectron.* **2016**, *76*, 164.
[16] L. Ge, S. Wang, X. Song, S. Ge, J. Yu, *Lab Chip* **2012**, *12*, 3150.
[17] a) S. Imani, A. J. Bandodkar, A. M. V. Mohan, R. Kumar, S. Yu, J. Wang, P. P. Mercier, *Nat. Commun.* **2016**, *7*, 11650; b) C.-C. Tseng, C.-T. Kung, R.-F. Chen, M.-H. Tsai, H.-R. Chao, Y.-N. Wang, L.-M. Fu, *Sens. Actuators B* **2021**, *342*, 130078.
[18] K. van Hoovels, X. Xuan, M. Cuartero, M. Gijssel, M. Swarén, G. A. Crespo, *ACS Sens.* **2021**, *6*, 3496.
[19] L. Rassaei, W. Olthuis, S. Tsujimura, E. J. R. Sudhölter, A. van den Berg, *Anal. Bioanal. Chem.* **2014**, *406*, 123.
[20] P. J. Derbyshire, H. Barr, F. Davis, S. P. J. Higson, *J. Physiol. Sci.* **2012**, *62*, 429.
[21] S. Rink, A. Duerkop, A. Jacobi von Wangelin, M. Seidel, A. J. Baeumner, *Anal. Chim. Acta* **2021**, *1188*, 339161.
[22] C. Marquette, *Biosens. Bioelectron.* **2003**, *19*, 433.
[23] R. M. Pino, J. Singh, *Anesthesiology* **2021**, *134*, 637.
[24] F. Li, J. Liu, L. Guo, J. Wang, K. Zhang, J. He, H. Cui, *Biosens. Bioelectron.* **2019**, *141*, 111472.
[25] A. Roda, M. Guardigli, D. Calabria, M. M. Calabretta, L. Cevenini, E. Michelini, *Analyst (Cambridge, U. K.)* **2014**, *139*, 6494.
[26] F. Li, X. Wang, J. Liu, Y. Hu, J. He, *Sens. Actuators B* **2019**, *288*, 266.
[27] a) D. Wang, C. Liu, Y. Liang, Y. Su, Q. Shang, C. Zhang, *J. Electrochem. Soc.* **2018**, *165*, B361–B369; b) M. L. Bhaiyya, S. Gangrade, P. K. Pattnaik, S. Goel, *IEEE Trans. Instrum. Meas.* **2022**, *71*, 1.
[28] A. L. Suherman, M. Lin, B. Rasche, R. G. Compton, *ACS Sens.* **2020**, *5*, 519.
[29] H. S. Toh, C. Batchelor-McAuley, K. Tschulik, R. G. Compton, *The Analyst* **2013**, *138*, 4292.
[30] J. Hu, S. Wang, L. Wang, F. Li, B. Pingguan-Murphy, T. J. Lu, F. Xu, *Biosens. Bioelectron.* **2014**, *54*, 585.
[31] J. Yu, S. Wang, L. Ge, S. Ge, *Biosens. Bioelectron.* **2011**, *26*, 3284.
[32] S. Kanchi, M. I. Sabela, P. S. Mdluli, Inamuddin, K. Bisetty, *Biosens. Bioelectron.* **2018**, *102*, 136.
[33] M. Mayer, S. Takegami, M. Neumeier, S. Rink, A. Jacobi von Wangelin, S. Schulte, M. Vollmer, A. G. Griesbeck, A. Duerkop, A. J. Baeumner, *Angew. Chem. Int. Ed.* **2018**, *57*, 408.
[34] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid et al., *Nat. Methods* **2012**, *9*, 676.

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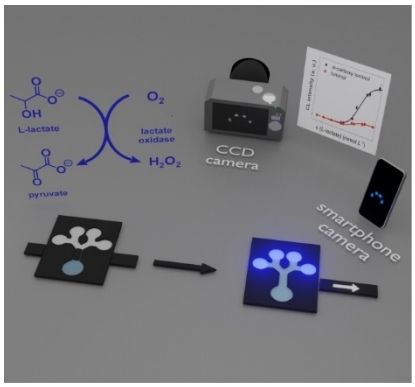
RESEARCH ARTICLE

A chemiluminescence μPAD employing a stronger emitting *m*-carboxy luminol for the detection of lactate in sweat is reported in this work, which directly compared the stronger luminophore with standard luminol and tested their performance when quantified with a smartphone camera as POCT detector and a scientific CCD camera as laboratory benchmark.



*S. Rink, Prof. Dr. A. Duerkop, Prof. Dr. A. J. Baeumner**

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Enhanced Chemiluminescence of a Superior Luminol Derivative Provides Sensitive Smartphone-Based Point-of-Care Testing with Enzymatic μPAD