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Bioaffinity-based assay for the sensitive detection and discrimination of sweat aimed at forensic applications

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ABSTRACT

Sweat is a well-known piece of biological evidence that is actually used much less than expected. Biological samples are important because their components can often provide some type of information about a person-of-interest. Sweat, in particular, is important because of its DNA content which can be extracted and analyzed to provide information that can be imperative to a criminal investigation. While it is a very important source of forensic information, the methods for detection and discrimination of sweat are limited, causing it to be overlooked during evidence collection. This manuscript presents a biocatalytic method for sweat detection that utilizes an enzyme cascade system that has the capability to detect trace amounts of sweat and distinguish it from saliva, even after the sample has dried. The results show the initial calibration studies performed to insure that the cascade performs well using both mimicked and authentic sweat samples which have components that could negatively affect the enzymes needed for the analysis. The method presented here also has the potential to be adapted for on-site analysis. The initial results of the development of a sweat-sensitive strip are shown here.

1. Introduction

Biological samples are imperative for many criminal investigations. [1] In particular, sweat can be an important source of evidence since it contains DNA [2–5] and other secretions [6–8] that could provide information about individuals present at a crime scene. Sweat glands are present throughout every part of the human body making it highly probable that individuals will leave traces of sweat every place they visit. [9,10] Sweat, however, is not always easily noticed or found, especially when dried, making it a piece of evidence that is often overlooked. [11] One of the main components of sweat that can be used for detection is lactate. [12–14] Lactate is produced when the human body breaks down carbohydrates for energy to create lactic acid which is then converted to lactate by various biochemical processes in the body. There are numerous methods for lactate determination and detection in samples, but few of the methods can be used for on-site forensic identification of sweat. The detection cascade presented in this submission is straightforward and we demonstrate that it can be used for quick, on-site discrimination of sweat from saliva. It was determined that the cascade can be used in a lab-based assay as well as with an on-site strip-based application that has been developed by us and is presented here. The detection of lactate is based on colorimetric properties and, therefore, gives a signal visible to the naked eye. Both mimicked and authentic sweat samples were analyzed along with authentic saliva samples. As shown, miniscule amounts of authentic sweat, as small as 50 nL, can be detected starting at 20 s. The cascade is also capable of detecting the sweat via lactate even after aging (drying) for a period of two weeks. Aged samples, samples left out in the environment for prolonged periods of time, were used to mimic crime scene scenarios where sweat is often left (and dried) in the environment for hours or days.

Sweat glands are present all over the epidermis of the human body and are constantly excreting fluids. Because of this, individuals will almost always leave some trace of their presence as they move from place to place.[9,10] Sweat is a common source of evidence in criminal investigations because it is a biological sample that yields DNA, [2,4,5] which can be used to directly link a specific individual to a crime scene. The DNA, however, can only be obtained if the sweat is found and collected at the scene. Depending on the amount of sweat left at the scene and the amount of time that has elapsed, it is possible that the sweat has dried and escapes the eyes of the investigators. This would lead to the loss of a valuable piece of evidence. This submission presents an enzyme-based cascade that makes the quick, on-site detection of sweat possible via the detection of lactate. Despite lactate having been used for applications involving health and fitness, methods for the detection of sweat via lactate have yet to be explored for use in

Abbreviations: Lox, Lactate Oxidase; HRP, Horseradish Peroxidase; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

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the field of forensic science. The detection of sweat for forensic applications is possible because there is a significant level of lactate present [15,16] and the high sensitivity of an enzyme cascade allows for the detection of a miniscule volume of sweat using a simple assay.

Various methods of lactate detection have been developed in the past, however, most of them require non-portable and complex instrumentation. For instance, in 1999, a photochemical method for the analysis of lactate was created using a flow-injection system. This method was dependent on the decomposition of the lactate sample in the presence of $UO_2^{2+}$and Fe^{3+}. [17] In the area of electrochemistry, a method using screen-printing carbon electrodes was used for detecting lactate. [18] An electrochemiluminescent biosensor has also been developed for lactate determination in saliva. [19] More recently, a group has created wearable tattoo biosensors for monitoring sweat using various components of its chemical makeup. [20–23] One of these studies focused on using lactate for monitoring sweat during exercise. [20] All of the current and aforementioned studies focus primarily on lactate as a physiological marker rather than a forensic marker. Sweat has been analyzed for a variety of health related purposes, but has yet to be used in crime related circumstances. The research presented here focuses on detecting and locating sweat as a marker for forensic applications instead of for the more commonly studied physiological applications described above.

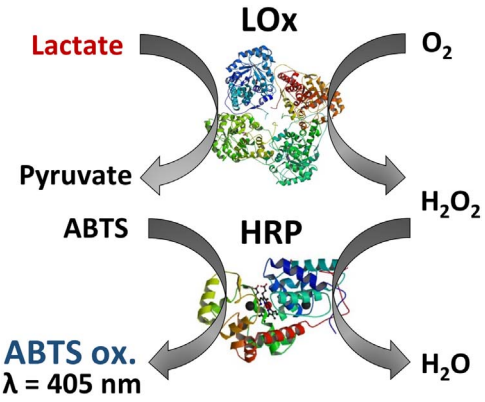
2. Materials and methods

Our investigation into the use of lactate for the analysis of forensic samples has led to the development of a purely biochemical method, a cascade-based bioassay, for the quick and sensitive detection of sweat via the presence of lactate. In this method, an enzyme selective for the recognition of lactate, lactate oxidase [24] (LOx; EC 1.13.12.4) is used. The unparalleled selectivity of LOx for lactate significantly decreases the potential of false-positive results and additional interference from other components in the sweat sample. This is an obvious advantage in comparison to established instrumental methods where the detection of small molecule-type analytes in complex mixtures of analogous compounds can be very challenging and often requires additional pre-cleaning/preparatory steps. This inevitably results in the necessity for more complex and time consuming analyses, which have led to the demand for straightforward, fast, and field-deployable methods for forensic applications. [25] Due to the simplicity of this method, complex instrumentation is not necessary and identification can be completed directly on-site. To prove the viability of this method, the assay was tested using both mimicked and authentic sweat samples. The sensitivity of the assay was tested with the use of various concentrations of lactate and volumes of authentic sweat. The sweat samples were also aged to show that detection is possible even after completely drying in an open environment. Furthermore, authentic sweat samples were tested along with authentic saliva samples to show that the method can discriminate between sweat and saliva. Lastly, an on-site application using a portable strip, a concept developed by our lab, was tested for use directly at a crime scene.

For the forensic detection of sweat, Scheme 1 shows the established cascade. The cascade consists of the enzymes lactate oxidase (LOx) and horseradish peroxidase (HRP; 1.11.1.7) in 0.1 M potassium phosphate (KH_2PO_4) pH 7.5 buffer. LOx consumes lactate along with O_2 to produce pyruvate together with H_2O_2. The H_2O_2 is subsequently used by HRP to drive the biocatalytic oxidation of the co-substrate ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). The oxidized form of ABTS provides an optical signal at 405 nm, [26] which is then quantitatively measured by a SpectraMax Plus384 Microplate Reader using polystyrene 96-well plates.

3. Results

In order to determine the ability of the enzyme cascade to detect



Scheme 1. The dual-enzyme cascade used for sweat detection where lactate is used to activate the cascade and drive the oxidation of ABTS. The abbreviations are as follows: LOx (lactate oxidase), HRP (horseradish peroxidase), and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). Information about the chemicals used is in the experimental section (ESI).

miniscule amounts of lactate, various concentrations of lactate dissolved in 0.1 M KH_2PO_4 buffer were tested. Each of the following concentrations of lactate was tested in triplicate: 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, and 0.1 μM. The average values obtained from the signals of each lactate concentration were plotted along with the corresponding error bars. Fig. 1A shows that the sensitivity of the cascade is capable of differentiating between the different concentrations of lactate. The three lowest concentrations did not generate significant signals and overlap with the negative controls, thus indicating that lactate is undetectable at such concentrations. Fig. 1B displays the response of the cascade when detecting different concentrations of lactate after 90 s of the reaction.

The composition of sweat, however, contains many additional components. [6–8] Therefore, it is possible that other components within the sample could affect the anticipated signal. To ensure the cascade-based assay would be viable for the analysis of authentic samples, sweat samples were obtained from a Caucasian female volunteer and tested using the cascade described above. All procedures were fully approved by and followed the regulations of the Institutional Review Board and Pre-Award and Compliance Office at the University at Albany, State University of New York. Informed consent was obtained from this volunteer by her signature on a provided document stating that there were no risks or benefits associated with this study, that there would be no payment for participation, and that no personal information would be used at any point during the study. The following volumes of sweat were tested in triplicate: 5 μL, 1 μL, 500 nL, 100 nL, 50 nL, and 10 nL. Fig. 2A shows the average signal obtained from the analysis of each volume of sweat. A significant signal is shown for 5 μL, 1 μL, 500 nL, and 50 nL. Fig. 2B shows the response of the cascade when detecting different volumes of authentic sweat after 90 s of the reaction.

In many instances, it is very probable that sweat and saliva are both found at a crime scene. To determine whether or not the sweat can be distinguished from another clear bodily sample, equal volumes of sweat and saliva were tested to determine distinguishability. Other bodily fluids can contain lactate and would provide a signal, but the signal would be minuscule and negligible as shown in Fig. 3 where 500 nL sweat and saliva samples were compared. Sweat contains much more lactate in comparison to other body fluids and would, therefore, provide a much larger signal as seen in Fig. 3. This information is valuable because various tests are available for different types of samples, making the knowledge that the sample is likely sweat useful. [27].

Also, since sweat is constantly left behind, the logical conclusion is that sweat present at a crime scene may not be fresh and has most

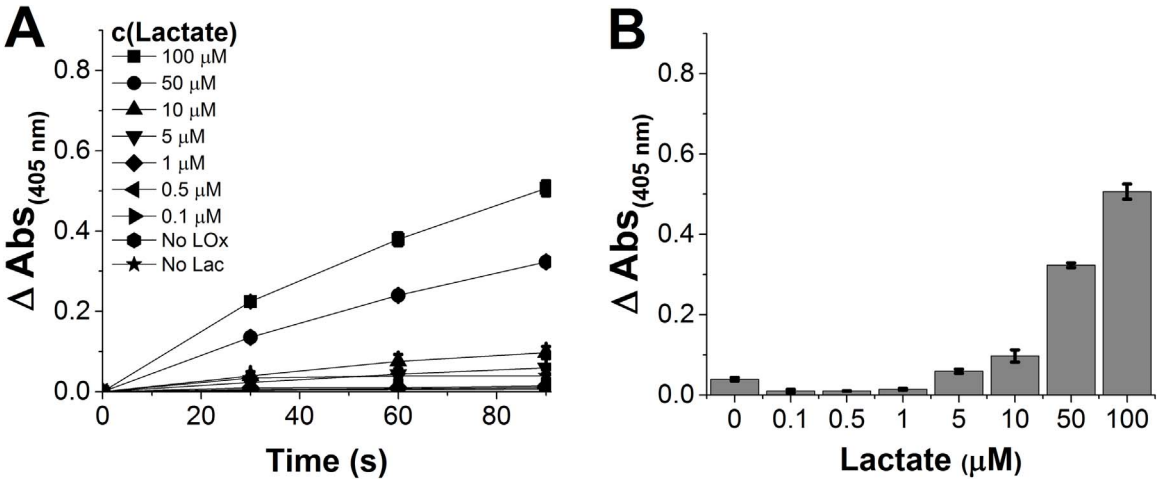


Fig. 1. (A) The change in absorbance (λ=405 nm) corresponding to the oxidation of ABTS as lactate samples are used to activate the cascade. These traces correspond to samples (n=3) containing the following lactate concentrations: 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, and 0.1 μM. The ESI contains information on all solutions used. (B) Signal obtained 90 s after initializing the assay. This diagram displays the absorbance at the time of the most significant difference between samples for more easily distinguishable comparison.

likely been present for days, especially if the crime was committed hours, days, or weeks prior to investigators collecting evidence. In order to test the efficiency of the cascade over time, aged authentic sweat samples were analyzed. A set of 15 samples containing 1 μL of authentic sweat were left to dry and then re-suspended for analysis; three samples were used for each day of aging. The components of the LOx-HRP cascade and the re-suspended samples were added into a well of a 96-well plate and the signal was recorded on the designated days. The samples were incubated at 25 °C (room temperature) and were tested on days 0, 1, 3, 6, 10, and 14. Fig. 4A shows the change in absorbance associated with the signal obtained from the assay for each day. Time zero corresponds to the analysis of the freshly prepared samples. As seen here, there is no significant change between the data collected each day, which shows that sweat can be detected with this method even after weeks of being left out in the environment. The bar graph, Fig. 4B, shows the change in absorbance of the assay at λ=405 nm after 90 s.

With the intention of ultimately creating a field deployable device for identifying sweat at a crime scene, we developed a portable paper strip sensor as a possible platform for the proposed concept of on-site analysis of lactate present in sweat. This will be useful in situations where it is obvious that a clear dried substance is present on a surface. This method is used in a similar fashion to a wipe test. In the initial development, presented here, paper strips were cut out and dipped into a polystyrene solution to make a surface for the immobilization of the necessary enzymes. This polystyrene coating was used to reproduce the

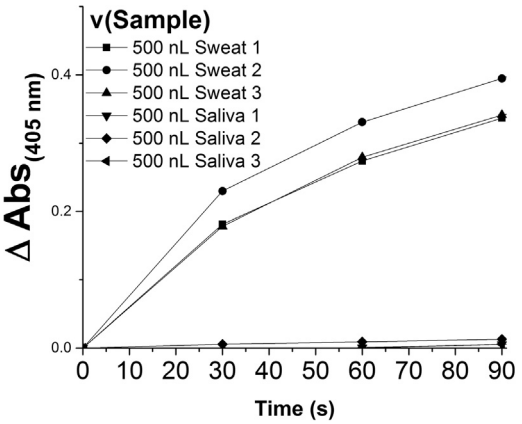


Fig. 3. The change in absorbance (λ=405 nm) corresponding to the oxidation of ABTS as sweat and saliva samples are used to activate the cascade. This illustrates that saliva samples contain low levels of lactate, but the concentrations are not high enough to induce a color change in the presented assay.

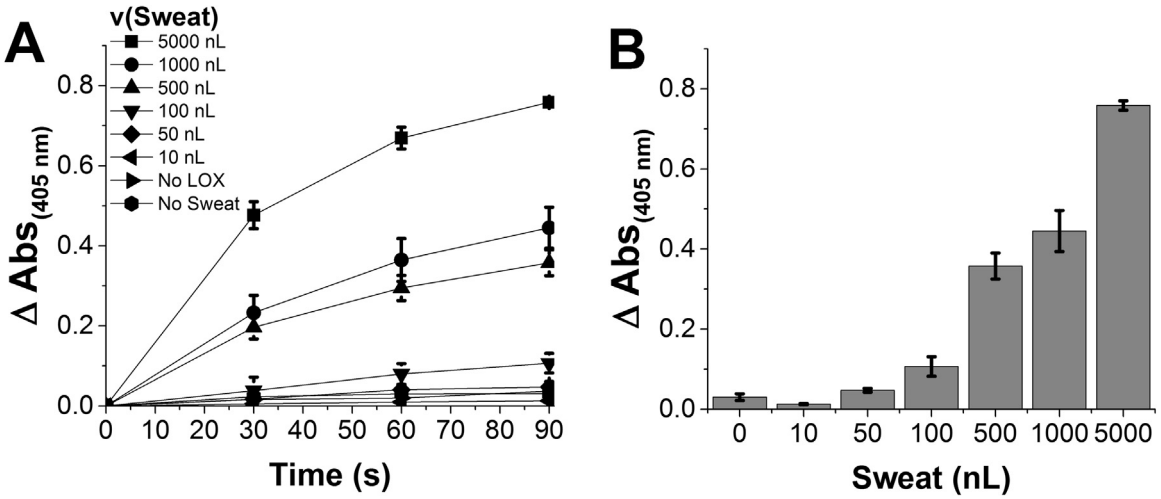


Fig. 2. (A) The change in absorbance (λ=405 nm) corresponding to the oxidation of ABTS as sweat samples are used to activate the cascade. These traces correspond to authentic sweat samples (n=3) for the following volumes: 5 μL, 1 μL, 500 nL, 100 nL, 50 nL, and 10 nL. The ESI has the exact composition of reactant solutions used. (B) Bar diagram representing the response of the system after 90 s. This diagram displays the absorbance at the time of the most significant difference between samples for more easily distinguishable comparison.

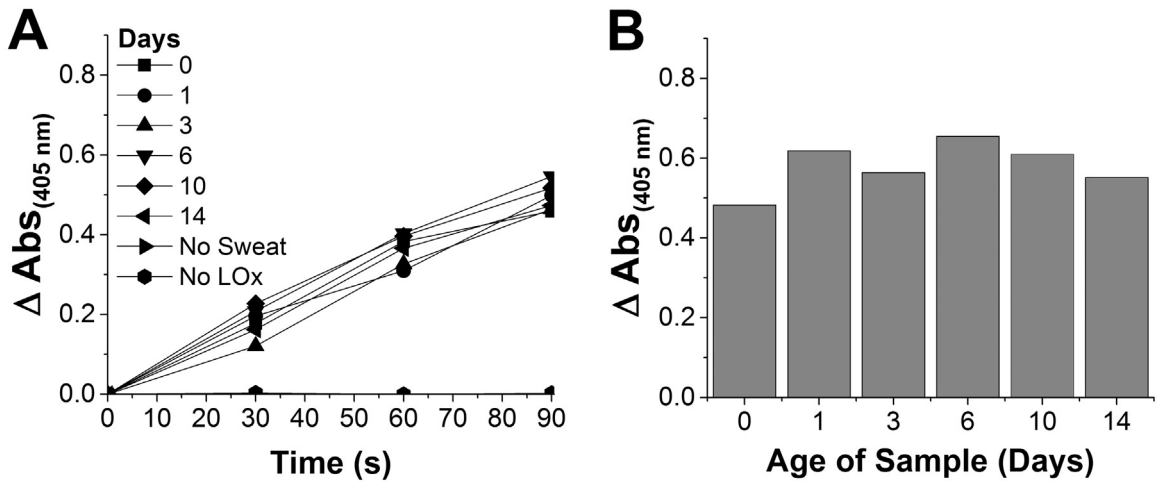


Fig. 4. (A) Absorbance change at λ=405 nm, corresponding to the oxidation of ABTS after the analysis of the samples, which underwent an aging process (up to 14 days), by the presented cascade-based assay. Samples were re-suspended in buffer after aging. There was no significant change in signal, indicative of the high stability of the sample. (B) Bar diagram representing the response of the system after 90 s. This diagram displays the absorbance at the time of the most significant difference between samples for more easily distinguishable comparison.

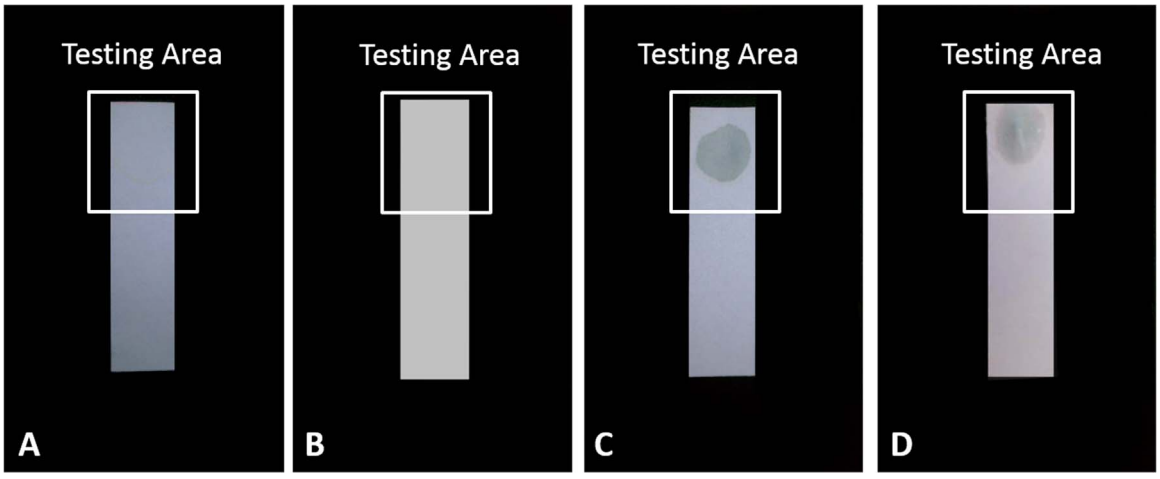


Fig. 5. Images of cascade-based assay on a paper strip. (A) Negative control strip photographed with no sweat used for activation. No change in color was expected as there was an absence of the analyte. (B) Control strip for a pure saliva sample. While trace amounts of lactate are reported in saliva, the concentration is not high enough to induce a color change. (C) Positive control strip photographed with re-suspended sweat sample used for activation. This strip shows the color induced by the activation of the assay by the sample's lactate component. (D) Positive control strip photographed with lactate sample used for activation.

adsorption capabilities of polystyrene plates that are commonly used in biotechnology. [28,29] Once the strips were dried, HRP and LOx were dissolved in KH_2PO_4 pH 7.5 buffer and deposited onto the strips, which were then left to dry overnight at 4 °C (specifics in ESI). The strips were then tested with 5 μL of sweat, lactate, and saliva samples. Each of the samples was left on a glass slide to dry and was subsequently reconstituted using ABTS dissolved in water. This was done in order to mimic the fact that most samples found at crime scenes are not freshly deposited. The strips were then swiped over the reconstituted samples and a camera was used to capture the resulting optical signals – observable by the naked eye after a minute. Fig. 4 shows four strips including the negative control strip and the strips used for testing saliva, lactate, and sweat samples with the cascade for on-site (portable) analysis. The negative control (Fig. 5A) is a strip without the presence of lactate, sweat, or saliva. The strip used to test saliva (Fig. 5B) shows no signal for 5 μL of pure saliva, which proves that even a concentrated amount of saliva would not provide a signal. The two positive controls show strips with the presence of 5 μL of a sweat sample (Fig. 5C) and 5 μL of a 10 mM lactate solution (Fig. 5D).

4. Conclusion

This investigation has shown that sweat can be detected biocatalytically using an enzyme cascade immobilized on a portable strip even when only a miniscule amount is present and the sample is up to 2 weeks old. The initial cascade analysis using samples consisting of only lactate dissolved in buffer showed the cascade's ability to detect lactate alone in amounts that are much lower than the significantly high concentration of lactate known to be present in sweat. To address the possibilities of authentic sweat samples not containing a sufficient lactate concentration for detection and the matrix effect negatively affecting the signals obtained by the cascade-based assay, authentic samples were tested using the same detection method. The testing of authentic samples also showed that various volumes of sweat can be detected using this method. In addition, to ensure that sweat can be detected after being dried in the environment, sweat samples were dried (aged) and tested over the course of several days. This showed that sweat can be detected using the presented cascade after 14 days without a significant decay in signal. The minimal decay in signal after 14 days sufficiently indicates that sweat can be detected via the lactate oxidase cascade for periods of time far surpassing the two weeks demonstrated here. Furthermore, it was proven that sweat can likely be

distinguished from other bodily fluids using the method in this manuscript.

This study has also proven that an enzyme-based cascade can be deposited onto a paper strip modified with polystyrene and used as a field-deployable device. While adjusting this system to work in an electrochemical strip [30] would give more definitive numerical data, the optical strip presented here is more simplistic and ideal for the binary response (absence or presence of sweat) that is desired for this system. As shown with the paper strip presented here, the identification of sweat can be performed without scientific training. This simple detection method is straightforward and generates a colorimetric signal that is visible to the naked eye, making it very easy to use by any and all members of law enforcement.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.04.016.

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