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Post deposition aging of bloodstains probed by steady-state fluorescence spectroscopy

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ABSTRACT

Blood is one of the most common body fluids discovered at crime scenes involving violent actions. It is one of the most important types of forensic evidence since it allows for the identification of the individual providing that there is a match with a known DNA profile. Determining the time since deposition (TSD) can assist investigators in establishing when the crime occurred or if a bloodstain present is actually related to the investigated event. To develop a forensically sound method for determining the TSD of a bloodstain, it is necessary to understand the underlying biochemical mechanisms occurring during aging. As biochemical processes occurring in blood are necessary for the continued survival of living organisms, they are important subjects of human biology and biomedicine and are well understood. However, the biochemistry of bloodstain aging *ex vivo* is primarily of interest to forensic scientists and has not yet been thoroughly researched. This preliminary study utilizes steady-state fluorescence spectroscopy to probe the changes in fluorescence properties of peripheral and menstrual blood up to 24-h post deposition. Peripheral and menstrual blood exhibited similar kinetic changes over time, assigned to the presence of the fluorophores: tryptophan, nicotinamide adenine dinucleotide (NADH), and flavins in both biological fluids. The biochemical mechanism of blood aging *ex vivo* is discussed.

1. Introduction

Peripheral blood is the most frequently found body fluid during investigations of violent crimes including: homicides, battery, and sexual assault [1]. Analysis of bloodstains can provide substantial assistance in crime scene investigations. The location of a bloodstain can help with the reconstruction of the event. Utilizing blood pattern analysis (BPA), the investigators can determine how the bloodstains occurred. Besides, once the bloodstain is identified, DNA analysis can be performed, which can be used to conclusively identify the individual of origin. Currently, several presumptive and confirmatory tests for blood detection are used. However, they are destructive, prone to false positives, and some tests cannot be done on site [2]. To overcome these issues new, universal, confirmatory, nondestructive, methods have been developed based on Raman spectroscopy [3–5] and ATR FTIR spectroscopy [6,7] for the identification of all main body fluids including blood. In addition, these methods allow for the discrimination between peripheral and menstrual bloodstains [6,8–12]. Raman spectroscopy is arguably the most developed emerging technology towards the practical forensic application since it has been already validated for analysis of bloodstains on various common substrates [13] and with respect to environmental interferences [14].

An additional form of analysis of bloodstains is to determine their time since deposition (TSD). By knowing the age of the bloodstain investigators are able to provide a time period in which the crime occurred. There are other numerous benefits to determining the TSD of bloodstains, it can assist investigators to determine which stains are relevant to the crime from those that are extraneous, aid in crime scene reconstruction, and be used to corroborate witness statements. Collecting only stains pertinent to the crime at hand creates more efficient processing of biological evidence and can assist in reducing and eliminating the large backlogs of evidence in crime labs. Creation of a method for determining TSD of bloodstains has been attempted since the 1900s [15–17], however, no single method has been adopted by the forensic community. There have been three extensive reviews that go in-depth on the aging process of bloodstains and the techniques thus far that have been created to determine their TSD [15,17], the most recent published by Weber and Lednev [16]. Techniques for determining the TSD of

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bloodstains have ranged from measuring the degradation of mRNA in white blood cells [18–23] to the analysis of racemic mixtures of amino acids using gas chromatography paired with mass spectroscopy [24]. However, based on the reported research, spectroscopic methods have become the leading techniques for the analysis of biological samples and for determining the TSD specifically. In one of the first reports on spectroscopy of bloodstain aging the Soret absorption band shifts were measured over time [25]. As a result, Hanson et al. were able to discriminate between stains deposited and aged for short periods (minutes to hours) *vs* those aged for extended time frames (days to weeks). More recent methods include the use of Raman spectroscopy which utilizes the measurement of inelastically scattered light to track the degradation in hemoglobin to determine a bloodstains age [26–29]. Attenuated total reflectance infrared (ATR FT-IR) spectroscopy which probes changes in amides [27,30] overtime. Finally, fluorescence lifetime measurements for the endogenous fluorophores [31–34] present in bloodstains were used for bloodstain dating. Understanding the biochemical mechanism of aging *ex vivo* should accelerate the method development for determining the TSD of bloodstains. We have recently utilized steady-state fluorescence for probing menstrual bloodstains' aging during the first 9 h post deposition [35]. The goal of this research is to expand that study to peripheral bloodstains.

The problem of creating a practical method to predict the TSD of bloodstains is complicated by a complex biochemical composition of blood and its potential variation including variation between donors and even within a singular donor based on the specific time/day of collection. Therefore, we expect some variations in quantitative characteristics measured during the aging process. The estimation of a confidence interval of the result is of high importance to forensic application. In addition, environmental conditions can have significant effects of the aging process. However, understanding the biochemical mechanisms under ambient conditions are required first, so testing varying environments is for future studies.

Peripheral blood accounts for approximately 8% of a human's body weight (12 pints) and has a complex chemical make-up. Blood is composed of red blood cells, white blood cells, and platelets all suspended in a matrix called plasma. Additionally within the plasma other components are present: including proteins, nucleic acids, lipids, and carbohydrates [2,16]. Biochemical processes, which occur in blood and play a vital role for living organisms, are well understood and are important subjects of human biology and biomedicine. However, the biochemistry of bloodstain aging *ex vivo* is primarily of interest to forensic scientists and is not as well understood now. It is important to attract attention of biochemical/biophysical community to this vital forensic problem.

When blood exits in the body, it is red in appearance, but as it dries, bloodstains darken. This red-to-brown color change is the result of the autoxidation of hemoglobin. As soon as blood dries, the red blood cells are broken. This releases the hemoglobin which is then oxidized into methemoglobin (metHb) by environmental oxygen. During this process, the ferrous iron in heme is converted into the ferric state. The additional conversion from oxyhemoglobin (oxyHb) to metHb is a continuous process that occurs as the blood ages. Once in the metHB state, the hemoglobin molecule is no longer able to bind oxygen. Finally, when blood is outside the body for an extended period of time, metHb will form aggregates of hemichrome [36–38]. During this process, the fluorescence of the bloodstains changes [26,39]. Within blood there are several different luminophores which are involved in biochemical reactions under aerobic conditions. A better understanding of these transformations during the blood aging after the deposition and their effect on bloodstain fluorescence was the goal of this study.

Within blood there are three main types of fluorophores that have been studied for understanding the aging mechanism: tryptophan, reduced nicotinamide adenine dinucleotide (NADH), and flavins (including flavin adenine dinucleotide (FAD)) [40,41]. Other endogenous fluorophores in peripheral blood include tyrosine, phenylalanine, and porphyrins [16], which are weaker in their fluorescent properties. Studies of bloodstain aging using fluorescence spectroscopy has primarily focused on measuring changes to the fluorescence lifetime of tryptophan [31,32,42]. Tryptophan exhibits a maximum emission peak at ~ 340 nm when excited at ~280 nm. Researchers reported promising results in attempting to determine bloodstain TSD based on fluorescence lifetime measurements, but thus far no conclusive methodologies have been developed. We denoted that significant changes occur in the steady-state fluorescence of menstrual bloodstains during the first nine-hours post deposition [35]. These results showed that the endogenous fluorophores in menstrual blood (tryptophan, NADH, and flavins) undergo monotonic changes as the result of the aging process. This allowed a deeper understanding of the underlying biochemical mechanisms that occur overtime.

The focus of the current study was to investigate peripheral bloodstain aging using steady state fluorescence spectroscopy. The observation time was extended up to 24 h post deposition that allowed for confirming the completion of the mono-exponential changes in tryptophan and NADH fluorescence. We noted that flavins' fluorescence was found to show less reproducibility and more complex behavior than tryptophan and NADH. Changes in the tryptophan and NADH fluorescence intensity showed different characteristic times indicating a complex biochemical mechanism of blood aging. The fluorescence properties of peripheral and menstrual bloodstains were compared, and the observed differences were discussed based on the difference in their biochemical composition. The proposed biochemical mechanism of blood aging is discussed and found to be applicable for both peripheral and menstrual bloodstains although some kinetic characteristics are different. Overall, this study reports on the fluorescent properties of peripheral and menstrual blood, as well as sheds light on the biochemical mechanism of blood aging *ex vivo* that is relevant to an important forensic problem, determining the time since deposition of a bloodstain.

2. Methods and Materials

2.1. Sample Collection

Samples were collected using the Institutional Review Board (IRB) approved protocol at the University at Albany. All blood donors participated in this study supplied written consent for the use of their blood for research purposes. This consent included the donors' acknowledgement that they were healthy, over the age of 18, and not using any prescription or recreational drugs and that they could withdraw from the study at any time without any repercussions.

Six fresh peripheral blood samples were obtained from one male Caucasian and one female African American donor, three samples from each donor. Blood samples were collected on different days, so that there was no storage prior to analysis. Before deposition, the aluminum foil-covered slides were cleaned using alcohol, deionized water, and thoroughly dried to remove any contaminants present on the slide. Approximately 20 μL blood was procured from the donor's cleaned finger, which was pricked using a safety lancet, and immediately deposited onto an aluminum foil-covered microscope slide. Aluminum foil was used as it does not possess any fluorescence interference within the spectral ranges tested. To compare the aging of menstrual blood samples with peripheral blood, five fresh menstrual blood samples were obtained from two Caucasian donors, three samples from Donor 1 and two samples from Donor 2. Samples were collected using a menstrual cup on the third day of the donors' menstrual cycles. Additionally, samples were collected during two different menstrual cycles for each donor: Donor 1 - two samples from the first cycle and one from the second, Donor 2 - one sample from each cycle. This collection protocol matches the protocol used in our previous menstrual blood study [35]. To measure the fluorescence of vaginal fluid, previously purchased commercial samples (LeeBioSolutions) were used. The vaginal fluid samples had been stored at −20 °C until time for deposition, at such time

they were thawed and homogenized using a vortex. 20 μLs of each sample was deposited onto a pre-cleaned aluminum foil-covered glass slide. The slides were then left to dry under ambient conditions (laboratory bench); the temperature and humidity were not specifically controlled.

2.2. Fluorescence Spectroscopy and Data Analysis

The slides were dried for one hour before being placed in the spectrofluorometer (FluoroLog 3, Horiba Scientific, Japan). The samples were held using a solid sample holder, situated at a 30-degree angle from the excitation beam to deviate the latter reaching the detector. The deposited bloodstains were larger than the excitation beam spot. The samples were subjected to fluorescence measurements at certain time points: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 24 h post deposition. The vaginal fluid samples were only analyzed at 2 h post deposition. Each spectral collection took approximately one minute. Samples were left in the instrument for the entire analysis time to ensure that the measurements were made from the same part of the dry stain. For each time point, emission spectra were collected after the excitation at 285, 350, and 465 nm and detection in the range of 295–525, 370–675, and 485–700 nm, respectively. Corresponding excitation spectra were collected for the emission wavelengths at 340, 460, 510, and 600 nm in the detection range of 200–320, 250–445, 300–490 nm, and 325–580 nm, respectively. The slits were set from 3 to 6 nm depending on the intensity of the fluorescence detected for the respective fluorophore within each body fluid. This was done so the detector was not saturated by high fluorescence intensity. The slits widths for the emission spectra of peripheral and menstrual blood were respectively: 2 and 3 nm (at 285 nm excitation), 3 and 5 nm (at 350 nm excitation), and 3 and 6 nm (at 465 nm excitation). The slit widths for the excitation spectra were 1 and 3 nm (at 340 nm emission), 3 and 5 nm (at 460 nm emission), and 4 and 6 nm (at 600 nm emission) for peripheral and menstrual blood, respectively. Vaginal fluid fluorescence spectra were collected within the same wavelength range, however, due to their strong fluorescence slit widths were set to 1.5 nm for previously noted excitation and emission spectral measurements. All spectra were imported into MATLAB (MathWorks, Inc.; version R2017b) with the PLS toolbox (Eigenvector Research, Inc.; version 8.9) for data treatment. Spectra with a poor signal-to-noise ratio were preprocessed using Savitzky-Golay smoothing with a 15 nm filter width. Data obtained was then analyzed in OriginPro 2019b (OriginLab Corporation, USA) to perform kinetic curve fitting.

3. Results

3.1. Fluorescence Spectra of Peripheral Bloodstains

Six total peripheral blood samples from two donors were aged up to 24-h post deposition and tested on the spectrofluorometer at 1, 2, 3, 4, 5, 6, 7, 8, 9, and 24 h. At each time point both excitation and emission spectra were collected to obtain a complete fluorescence profile of the sample. The parameters to measure menstrual blood's fluorescence were used to determine the wavelength pairs in this work [35]. Each fluorophore had a unique excitation/emission wavelength pair, 285/340, 350/460, and 465/600 nm. As the excitation spectra did not overlap, it was concluded that there were three different endogenous fluorophores within peripheral blood in the investigated spectral range. The excitation and emission peaks were consistent with the previous study involving menstrual blood [35]. As a result, the peaks were able to be correlated to the same endogenous fluorophores, which were previously assigned based on an extensive literature review of fluorescent components found in peripheral blood [31,32,41–44]. The excitation/emission wavelength pair of 285/340 nm was assigned to the first fluorophore - tryptophan. Tryptophan is an aromatic amino acid which is present in numerous blood proteins [31,32,42]. Tyrosine's fluorescence quantum yield is lower than that of tryptophan [45]. In addition, Tyrosine has the emission maximum at 304 nm when excited at 200–220 nm [45]. As a consequence, the fluorescence of tyrosine would not interfere with the spectra reported here. The spectral peaks associated with the 350/460 nm excitation/emission wavelength pair corresponded to NADH. This is an electron transporter that is fundamental to the energy production process within the mitochondria [44,46]. Finally, the 465/600 nm wavelength pair was assigned to the group of fluorophores called flavins. There are several flavin types including: riboflavin, the phosphorylated form – flavin mononucleotide (FMN), and the form attached to adenosine diphosphate – flavin adenine dinucleotide (FAD). FAD is the most common form of flavin found in blood as it is responsible for electron transportation within human cells [44,46,47]. All forms of flavins have a similar fluorescence profile as they contain the same main group – pteridine, which is a conjugated compound formed by the tricyclic heterocycle isoalloxazine [44].

When plotted, the fluorescence spectra of each fluorophore in peripheral blood showed significant monotonic changes over the first 24-h post deposition. These intensity changes included a decrease of the tryptophan peaks and an increase of the NADH peaks over time. The flavin peaks presented a more complicated trend, a two-stage process that first involved an intensity decrease in the first three hours, followed by a large increase in the remainder of aging trial. The peak positions remained relatively consistent, with only the NADH maximum wavelength in the excitation spectra shifting slightly throughout the experiment (see supplemental materials). However, the relative intensity for each of the fluorophores differed significantly. There are a number of factors that can account for these differences including the variable concentration of the fluorophores, the extinction coefficient, and the fluorescence quantum yield of the respective fluorophores, as well as the intensity of the exciting beam, and the overall spectral sensitivity of the detector including the throughput of the collecting optics and diffraction grating. Therefore, to account for this disproportion between fluorescence intensities, different slit widths were used for each fluorophore (see Experimental section for details), as to not overload the detector. The spectra presented in this article did not undergo any normalization nor were they corrected for the spectral characteristics of the light source or detector. The comparison of the excitation and emission spectra collected over 24-h post deposition from Sample A, obtained from Donor 1, with an indication of the direction of predominant intensity changes, is presented in Fig. 1. The spectra were graphed starting at the second hour, as during the first hour the peripheral blood samples were not completely dried, which could affect the fluorescence characteristics. Data obtained from the other samples from Donor 1, as well as the results obtained from Donor 2 (see Supplementary Fig. S1), showed similar intensity changes in the fluorescence profiles.

3.2. Comparison of Peripheral and Menstrual Bloodstains' Fluorescence

To determine how peripheral blood samples age compared to menstrual blood, fresh menstrual blood sample were also collected, aged, and analyzed up to 24 h. The obtained results showed significant similarities between peripheral and menstrual blood fluorescence spectra and a complete agreement with menstrual blood data reported up to 9 h post deposition [35]. However, some differences were also denoted between the peripheral and menstrual blood samples. When deposited, peripheral blood had a uniform viscous consistency and were red in color that converted into red-brown during the aging process. However, this was not the same for menstrual blood. The menstrual blood samples varied in their viscosity, while some were "thin" in their consistency others appeared closer to peripheral blood in their viscosity. This was attributed to the composition of menstrual blood, having both blood and vaginal fluid components. These differences affected the color of the menstrual blood samples as well, being lighter in color than the peripheral bloodstains.

To further assess the fluorescence characteristics of these two biological samples, the emission spectra for the three fluorophores of

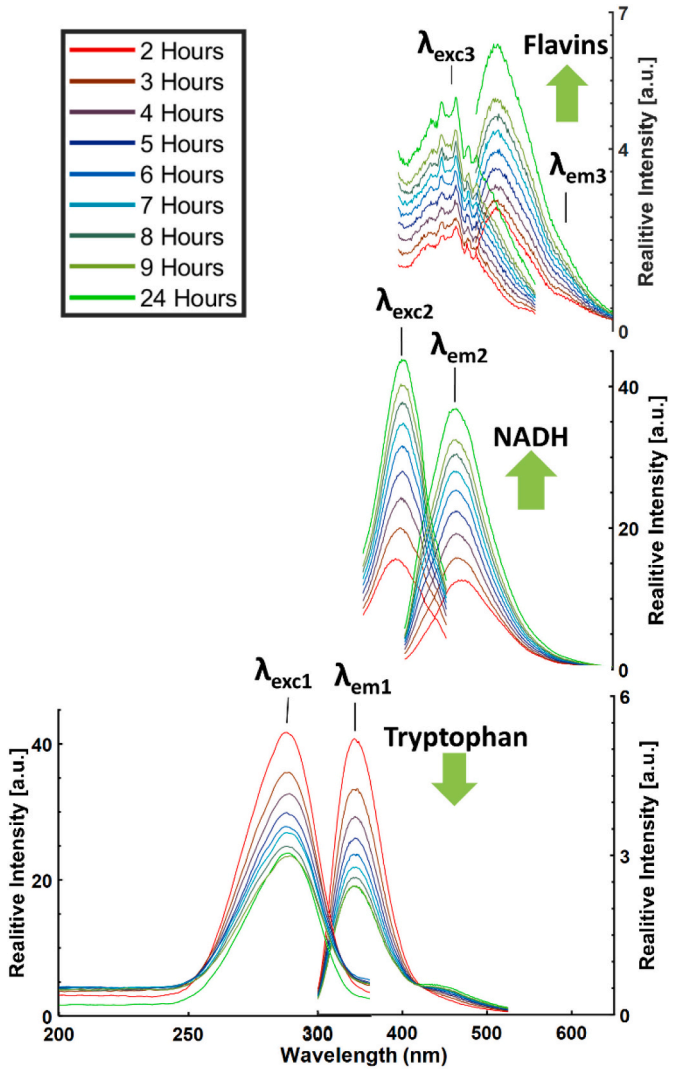

Fig. 1. Changes in the peripheral bloodstain fluorescence with time since deposition. Excitation and emission spectra collected over first twenty-four hours after deposition of one peripheral blood sample obtained from Donor 1. Black lines indicate the values of excitation wavelengths applied to measure emission spectra, respectively $\lambda_{exc1} = 285$ nm, $\lambda_{exc2} = 350$ nm, $\lambda_{exc3} = 465$ nm, and emission wavelengths fixed for the collection of excitation spectra, respectively $\lambda_{em1} = 340$ nm, $\lambda_{em2} = 460$ nm, $\lambda_{em3} = 600$ nm. Green arrows indicate the direction of intensity changes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interest were compared between the peripheral and menstrual blood samples as shown in Fig. 2. A "composed" emission spectrum was obtained for each individual sample by combining emission bands resulting from the three emission/excitation conditions onto one spectrum. Each of these bands was acquired under specific excitation parameters including the excitation wavelength and slits' width. The intensity of each band depended on the lamp and detector characteristics as well as monochromator and the selected slits. It is important to mention that all three emission bands were obtained from the same spot on the sample and all excitation/emission conditions were kept consistent within each class of samples: peripheral blood, menstrual blood, and vaginal fluid. The slit widths could not be standardized between classes, as each biological sample exhibited varying intensities of fluorescence when excited at the specific wavelengths. The vaginal fluid samples required the smallest slit widths. While kept the amount of sample deposited consistent the differences in fluorescence intensity might be the result of a more efficient excitation conditions because the vaginal fluid stain was transparent for the excitation light in contrast to bloodstains. In contrast, menstrual blood required the largest slit widths since these samples exhibited the lowest fluorescence intensity. As all parameters were unable to be standardized, we are only able to compare between each of the other composed emission spectra qualitatively or in terms of variability.

The presented spectra are from the second hour post deposition, so the biological samples were compared with the least amount of aging influence. For each type of blood, we chose samples for Fig. 2, which showed the largest difference in relative intensity of tryptophan and NADH emission peaks to illustrate the variation observed for samples of body fluids of the same type. The peripheral blood samples, Fig. 2A and B, show the most consistency of relative peak intensity between fluorophores, with tryptophan having the highest intensity, followed by NADH, and ending with flavins. While there is some variation within the relative intensity between tryptophan and NADH for peripheral blood, when compared to the menstrual blood samples, the changes are minor. The menstrual blood samples, Fig. 2C and D, show a large amount of variation between trials. The relative intensity of the tryptophan to NADH peak differs significantly between trials, with the NADH peak sometimes having a substantially larger intensity. Even in one trial the measured intensity of the NADH peak was almost equal to the tryptophan peak (see 2C). Additionally, the flavin emission spectra displayed a difference in the fluorescence profile for the menstrual blood samples, having a low intensity peak at 600 nm much more apparent than that for the peripheral blood samples. Previously, this fluorescence peak was tentatively assigned to the endogenous porphyrins of hemoglobin in blood [41,43]. Though the excitation spectra collected at 600 nm did lack the characteristic porphyrin band around 400 nm, it did have a band around 465 nm, consistent with flavin excitation [41,43,44].

The differences between peripheral and menstrual blood samples, from the color of the sample upon deposition as well as the relative peak variability, caused question into the fluorescent properties of the other main component of menstrual blood, vaginal fluid. Three previously purchased commercial samples of vaginal fluid were tested two hours post deposition. A time trial was not performed on these samples as they were not freshly collected and had been frozen for an extended period, which could affect the biochemical mechanisms that would naturally occur during the aging process. The obtained results, presented in the composed spectra in Fig. 2E and F, indicate that vaginal fluid possess similar fluorescent components as peripheral and menstrual blood. This may indicate that vaginal fluid also contributes to the fluorescence signal of menstrual blood. Additionally, the vaginal fluid samples do not possess a peak around 600-nm, which was tentatively attributed to the endogenous porphyrins within hemoglobin [35,41,43]. This confirms that the peak present at 600-nm in menstrual blood fluorescence spectrum is due to the blood component rather than vaginal fluid.

3.3. Kinetics of Bloodstain Fluorescence Changes

In order to characterize the aging kinetics, the maximum intensity of emission peaks for the fluorophores (tryptophan, NADH, and flavins) were plotted *vs* time for both peripheral and menstrual blood, shown in Fig. 3. Plotting the fluorescence intensity in terms of their maximum intensity allows for a visual representation of how the fluorophores changed post deposition. Kinetic plotting and curve fitting was done from 2 to 24 h post deposition. The first hour was excluded for consistency, as the samples of peripheral blood were still wet, which could affect the fluorescent readings. A mono-exponential decay function, described by the equation $y = A \exp.(-x/t) + y_0$, was used to fit the kinetic trends. The fitting was good for the majority of the samples, having R^2 values of more than 0.99 for the tryptophan and NADH curves. In compliment to the mono-exponential curves (Fig. 3), logarithmic functions were plotted to display the intensity changes in a linear form (see Fig. S4 in supplemental material).

The obtained values of time constant "t" were averaged for both

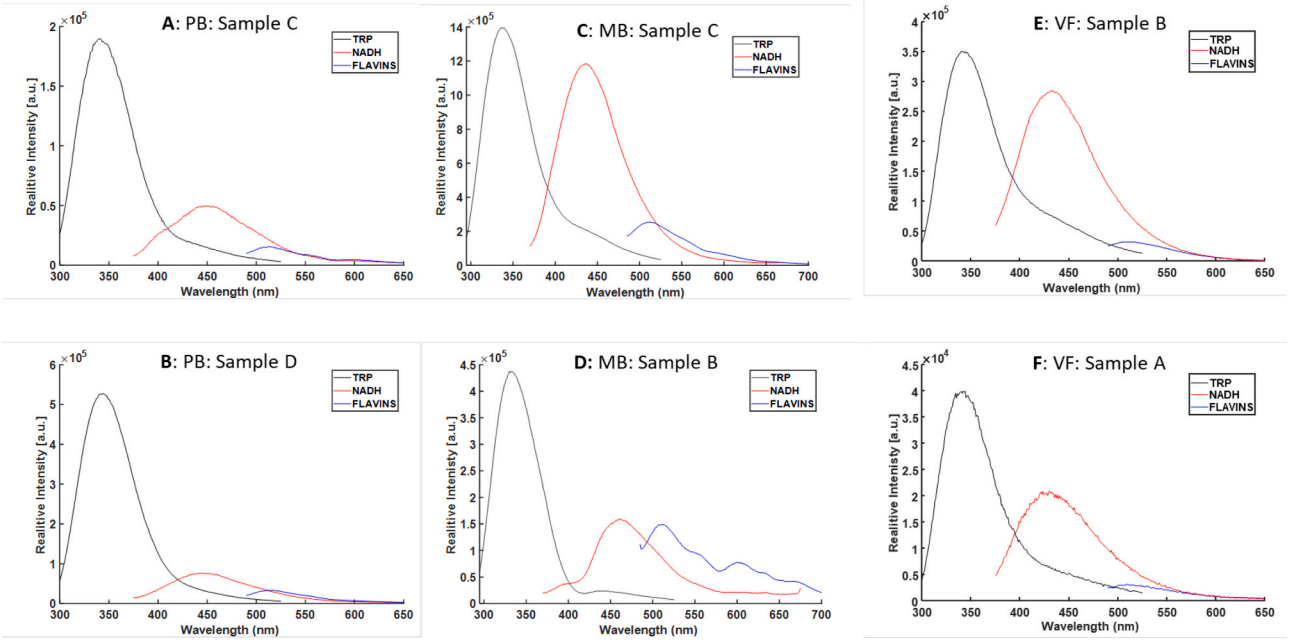

Fig. 2. Fluorescence spectra of bloodstains two-hour post deposition demonstrating higher consistency of peripheral blood samples than that of menstrual blood. Composed emission spectra collected from peripheral (A and B) and menstrual blood (C and D) samples obtained from different donors at different days. Samples demonstrating the largest variability in fluorescence properties are shown. Vaginal fluid (E and F) fluorescence is shown for comparison. According to our assignment, these spectra depict the emission of tryptophan (black peaks), NADH (red peaks), and flavins (blue peaks). The wavelengths of excitation are $\lambda_{Tryptophan} = 285$ nm, $\lambda_{NADH} = 350$ nm, $\lambda_{Flavins} = 465$ nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

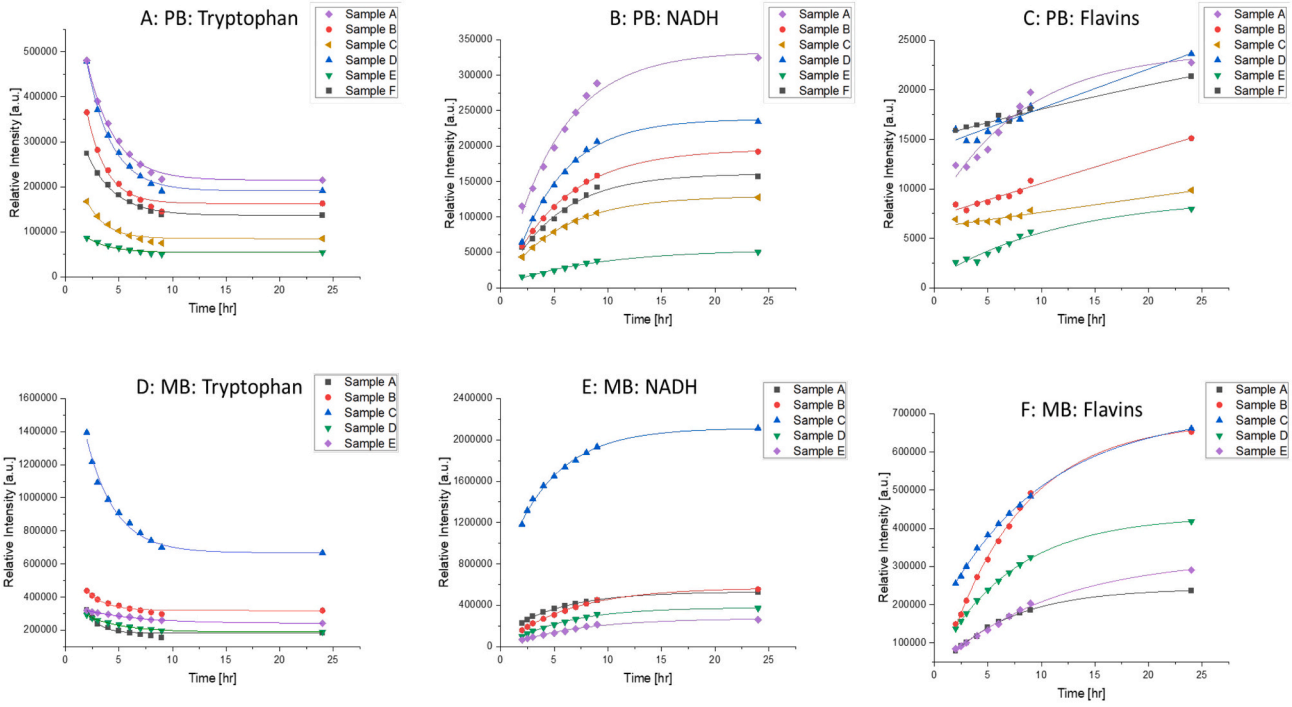

Fig. 3. Kinetic intensity changes of the fluorescence emission peaks for the fluorophores (Tryptophan, NADH, and Flavins) of both peripheral (top row) and menstrual (bottom row) blood. Each curve depicts an individual sample during aging: six samples for peripheral blood and five samples for menstrual blood. All samples were measured within the first twenty-four hours after deposition. The intensity value for the maximum wavelength was graphed *vs* time and fitted with mono-exponential functions, except for Samples B, C, D, and F in 3C. These samples did not reach saturation and could not be fitted with a mono-exponential function. The wavelengths of excitation are $\lambda_{Tryptophan} = 285$ nm, $\lambda_{NADH} = 350$ nm, $\lambda_{Flavins} = 465$ nm.

Table 1
A characteristic time for the fluorescence intensity change found for each fluorophore in peripheral (PB) and menstrual (MB) bloodstains.

Sample	t_{avg}[hr]	Absolute error (95% CI)
PB: Tryptophan	2.5	0.3
MB: Tryptophan	3.0	2
PB: NADH	5.8	2
MB: NADH	5.6	1
PB: Flavins	10 and longer	3
MB: Flavins	8	2

peripheral and menstrual blood and presented in Table 1 with a statistical error estimated for the 95% confidence interval (CI). The characteristic time constants for tryptophan were 2.5 ± 0.3 h and 3.0 ± 2 h for peripheral and menstrual blood, respectively. Similarly, the time constants for NADH were 5.8 ± 2 h (peripheral blood) and 5.6 ± 1 h (menstrual blood). The characteristic times for peripheral blood tryptophan and NADH kinetic curves do not overlap within the error, which indicates that two different reactions occur involving these luminophores during the aging process. This observation cannot be expanded to menstrual blood now, where the characteristic times do overlap within the error based on the current data, which is tentatively attributed to the variability within the sample composition.

The kinetic trends for the flavin curves were more challenging to analyze, as there was significant variability in the results. For the peripheral blood samples, only two of six trials (Samples A and E in Fig. 3C) presented a mono-exponential trend with the average characteristic time of 10 ± 3 h. The remaining samples (B, C, D, and F) did not reach saturation and exhibited a linear increase over time indicating a much longer biochemical process. In contrast, while the mensural blood samples had varying results between trials, all of the samples presented a monoexponentially increasing trend and appeared to be approaching saturation. The averaged characteristic time constant of flavins for menstrual blood was 8 ± 2 h.

4. Discussion

4.1. Biochemical Mechanism of Bloodstain Fluorescence Changes During Aging

When compared to the menstrual blood fluorescence spectra detailed in our previous article [35], the current data obtained for peripheral blood (Fig. 1) show that both body fluids exhibit similar kinetic changes overtime. These changes were attributed to the aerobic aging of the bloodstains *ex vivo* and are summated schematically in Fig. 4 and Fig. 5. Since peripheral and menstrual blood show similar fluorescence properties and overall kinetic changes, it is anticipated that similar biochemical mechanisms should occur during aging. Aging in an ambient environment exposes the bloodstains to aerobic conditions, due to the excess amounts of O_2 present. In this environment, free tryptophan amino acid, and the bound tryptophan protein residues, such as in

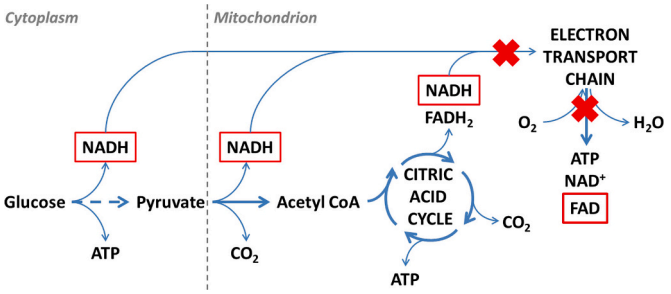

Fig. 5. Schematic representation of cellular respiration *ex vivo*. Fluorescent compounds are marked with red. Red crosses indicate processes that are stopped first under aerobic conditions. Reprinted from Wójtowicz, A. et al., Probing menstrual bloodstain aging with fluorescence spectroscopy. *Spectrochim Acta A Mol Biomol Spectrosc*, 2021. 248: p. 119172. (ref no. 35) Copyright 2020, with presmission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hemoglobin and human serum albumin, undergo oxidation, shown in Fig. 4. This process transforms the fluorescent molecule of tryptophan into the product, kynurenine, which has a lower fluorescence yield (0.005) than tryptophan (0.13) [48]. The excitation/emission characteristics of kynurenine is known from the literature: kynurenine absorbs light at 330 nm and 380 nm emitting at 480 nm [48,49].. However, no noticeable fluorescence of aged bloodstains was found to be consistent with these characteristics most probably because of kynurenine's low fluorescence quantum yield. Thus, as bloodstains are oxidized during aging, the fluorescence intensity of tryptophan decreases as the concentration of tryptophan diminishes.

In vivo, red blood cells are significant suppliers to energy production, contributing to both glycolysis and the Krebs cycle during cellular respiration. Cellular respiration is the process in which energy is created by the transfer of electrons from NADH and $FADH_2$ (the reduced form of FAD) to O_2 by a series of electron carriers within the electron transport chain. During this metabolic process, the body produces ATP, NAD^+, FAD, and water which allow for continued function. This process occurs within the cytoplasm and mitochondria of red blood cells. Our experimental observations were consistent with a literature scheme (Fig. 5) indicating that there was an excess of the reduced form of the transporters NADH and $FADH_2$, accumulated from glycolysis and the Krebs cycle after exposure to an aerobic environment [35]. This is tentatively attributed to the possibility that the metabolic demand is halted *ex vivo* leading to a break-down of the electron transport chain [46,50,51], depicted in Fig. 5. However, since the reduced form of NADH is fluorescent, the increase in its concentration leads to an increase in fluorescence as the bloodstains age. It was noted that, like in the menstrual blood data, there was a shift in the NADH emission peak at 350 nm during aging. This was attributed to the fact that the emission λ-max of NADH is dependent on the environment of the emitting component [44]. Additionally, Achetibet al. have analyzed the fluorescence of another

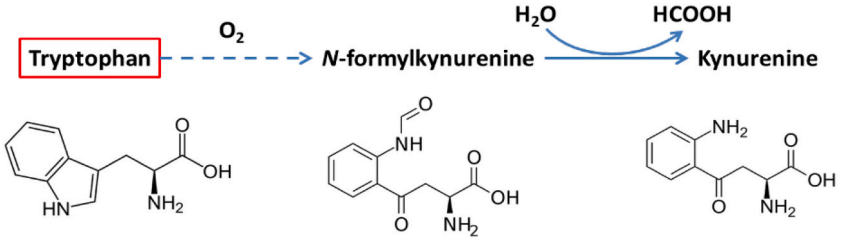

Fig. 4. Oxidation of tryptophan *ex vivo*. The primary fluorescent compound is marked with red. Reprinted from Wójtowicz, A. et al., Probing menstrual bloodstain aging with fluorescence spectroscopy. *Spectrochim Acta A Mol Biomol Spectrosc*, 2021. 248: p. 119172. (ref no. 35) Copyright 2020, with presmission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biological fluid, semen, at 283 nm and 365 nm excitation. They assigned the 283-nm excitation peak to the fluorescence of a broad group, proteins. While the 365-nm excitation peak was assigned to 'fluorescent oxidative products (FOX)' which are comprised of a combination of lipids and proteins [52]. Achetibet al. found that as the protein emission decreases, the FOX emission increases, stating that these phenomena are related [52]. This is different than other literature studies of blood which indicate these peaks are caused by two different fluorophores tryptophan and NADH, respectively [41,43]. Though these fluorescence peaks have similar excitation/emission wavelengths, they could be due to different species in different biological fluids, thus further investigation is needed to clarify this issue.

In contrast to NADH, the oxidized form of FAD is fluorescent. When measuring the peripheral bloodstains there is an initial decrease in the fluorescence intensity of flavin emission peak during the first three hours post deposition (see Fig. 3 for details). This is consistent when factoring in the proposed "breakdown" of the electron transport chain, FAD is no longer being produced, leading to a decrease in its concentration and subsequent fluorescence intensity. However, after this brief decrease there is a significant increase in intensity for the remainder of the time trial. These nonmonotonic changes indicate that multiple biochemical processes should occur. Since the excitation and emission spectra do not change noticeably with time, we can assign tentatively the observed spectra to the same or similar fluorophores, FAD and other flavins specifically. This preliminary assignment is based on the fact that similar to FAD, the oxidized forms of other flavins fluoresce [47]. After a prolonged exposure to an oxidized environment, there is an increase in the concentration of these fluorophores, leading to an increase in their emission. In other words, one tentative explanation is that the changes in the fluorescence of flavins of peripheral bloodstains, as with menstrual bloodstains, is a two-stage process: (1) a decrease in intensity within the first 3-h due to the breakdown of the electron transport chain leading to a lack of FAD and (2) an exponential increase in the intensity caused by the oxidation of additional flavins present in bloodstains [35]. In our previous paper, we also speculated that the nonmonotonic changes may be attributed to the drying process and changes in fluorescence because of it [35]. This may still be a contributing factor. Further research, focusing on the early periods post deposition, are needed to understand how the initial drying process can affect blood fluorescence.

4.2. Biochemical Comparison of Peripheral and Menstrual Blood

Peripheral and menstrual blood 'composed' spectra were qualitatively compared at 2 h post deposition. A large number of similarities were observed between these two body fluids. When compared with observations noted in our previous manuscript [35] it is apparent that both peripheral and menstrual blood possess similar endogenous fluorophores that undergo similar kinetic trends during the post deposition aging. However, some differences between their behaviors were noted as well. It was observed that the fluorescence of menstrual blood had substantial irregularity between relative peak intensities for different samples. This variation was attributed to complex biochemical composition of menstrual blood. Menstrual blood is a biological fluid composed of peripheral blood, cervico-vaginal secretions, endometrial tissue, epithelial cells, and vaginal microbes [53,54]. Additionally, cervio-vaginal secretions have their own complex composition, which exhibits changes based on a woman's menstrual cycle and varies based on sex steroid levels. Vaginal secretions are composed of vulvar secretions from sebaceous glands, exfoliated cells, cervical mucus, and endometrial and oviductal fluids [55]. Though there are a number of additional components, when compared to peripheral blood, there are also substances which amount is significantly diminished in menstrual blood relative to peripheral blood. Specifically, to ensure a healthy expelling of menstrual fluid, the blood must maintain a low viscosity. Menstrual blood is able to maintain a more liquid form as it does not contain significant amounts of the proteins hemoglobin, prothrombin, thrombin, and fibrinogen which allow blood to clot [56]. The lack of these proteins may affect the concentration of the endogenous fluorophores as well as the macroscopic differences in color apparent to the naked eye when deposited. Additionally, to ensure clotting does not occur, menstrual blood has a lower concentration of iron and blood platelets. While the concentration of proteins is unique to each body fluid, the numerous components of menstrual blood create an environment where the amounts of proteins vary on a daily and monthly basis. There are numerous factors that can affect the chemical composition of a woman's menstrual secretions. This includes the composition of vaginal secretions, which are not a concern when measuring the fluorescence of peripheral blood.

4.3. Future Uses and Complexity of Fluorescence Spectroscopy of Bloodstains

When comparing peripheral and menstrual blood t_{avg} values, the classes are indistinguishable as the corresponding t_{avg} values overlap within the 95% confidence interval, further indicating that the biochemical mechanisms occurring during aging are similar for both bodily fluids. The kinetic trends for the tryptophan and NADH peak intensities show the most consistency between trials. Indicating that the tryptophan and NADH are most useful to characterize the kinetic changes occurring within bloodstains overtime. Therefore, when expanding this research to determine the time since deposition (TSD) of bloodstains, these two peaks could be used to create an aging model.

Characterizing the aging of bloodstains is challenging to accomplish, as there are numerous parameters that affect the way that blood ages post deposition. These complex parameters begin at the composition of the bloodstain, which research has shown to be donor dependent. Other parameters that affect aging include, but are not limited to, temperature, humidity, and sunlight exposure. As these conditions are difficult, or impossible in the case of blood composition, to control, creating a method to estimate time since deposition of bloodstains is a laborious task. To some extent the investigation of bloodstain aging is complicated by the dependence on the donor. This is similar to biomedical research when testing human responses to drugs. These studies typically require pilot clinical trials to account for all possible variations of response within donors. As this is a proof-of concept study, we did not have a donor size near that large, but we do note that due to the variations of blood composition, both between and within donors, further investigation is required before this research is capable of being used to quantitatively determine the time since deposition of bloodstains..

4.4. Road-Map to Develop a Usable Forensic Method

This proof-of-concept study, measuring the changes to the fluorescence of peripheral and menstrual blood, is a starting point towards developing a quantitative method that is able to determine the TSD of bloodstains. We have shown that there are measurable changes in bloodstain fluorescence overtime. However, there is significantly more research required before one can create a quantitative model that is capable of estimating the TSD of peripheral and menstrual blood. Here we list a number of items that need to be addressed to advance this preliminary work into a usable method for bloodstain age analysis. This list is not exhaustive, we expect that as we further investigate the aging of bloodstains, more specific questions will arise.

(1) Investigate thoroughly the biochemical mechanism of bloodstain aging.

- Investigate model systems including individual luminophores in buffered solutions.
- Conduct bloodstain aging in anaerobic conditions.

- Conduct lifetime measurements for individual fluorescence peaks to further validate the assignment to a specific luminophore.

(2) Develop and validate a forensic method for determining TSD based on fluorescence analysis of a bloodstain.

- Significantly increase the number of donors and their diversity based on sex, race, and age.
- Increase the number of time points for testing and extend the analysis beyond 24 h.
- Analyze the fluorescence of a single sample at multiple sections to determine the variation within a single bloodstain; investigate the effect of bloodstain thickness.
- Test external parameters that may affect aging including, but not limited to, temperature, humidity, and sunlight exposure.
- Test the effect of common substrates.
- Utilize chemometrics for data analysis to establish statistical significance of the established kinetic trends. Develop a regression model for determining the TSD of a bloodstain.

5. Conclusion

In conclusion, this study characterizes the aging trends of peripheral blood up to 24-h post deposition utilizing fluorescence spectroscopy. Very significant monotonic changes in blood fluorescence spectra were assigned to the components tryptophan, NADH, and flavins. When compared to the aging of menstrual blood, it was revealed that peripheral blood exhibits the same fluorescent behavior during the aging process. These changes include a similar change in the oxidation of tryptophan, build-up of NADH, and an initial deficiency of FAD followed by an increase in flavins through oxidation in both sample types [35]. To depict the post-deposition changes, peak maxima of the fluorescence intensities of corresponding fluorophores during aging were plotted *vs* time and fitted with kinetic curves. The kinetic curves and their respective characteristic times were reported for both peripheral and menstrual blood. The kinetic trends for the fluorophores up to 24-h were consistent with previous results reported for menstrual blood samples for 9-h post deposition [35]. As peripheral and menstrual blood possess similar fluorophores, their fluorescence properties are not expected to be significantly different. It was shown that peripheral and menstrual bloodstains are indistinguishable at a qualitative and kinetic level based on their fluorescent properties. This is depicted by their analogous emission spectra and characteristic times for kinetic changes, respectively. However, there were some noticeable differences detected between the emission spectra of the peripheral and menstrual blood samples. The NADH and flavin's emission peak shape varies between samples, showing the complexity of the biochemical mechanisms within bloodstains.

Additionally, the menstrual blood samples exhibit more compositional variability than peripheral blood, with the relative intensities of tryptophan to NADH in the composed spectra depicting the largest variability at the earliest time point post deposition. Qualitatively, the tryptophan to NADH peak intensities for menstrual blood is closer to that of vaginal fluid than peripheral blood. This can be tentatively correlated to composition of menstrual blood, including the presence of vaginal fluid and the different concentrations of proteins when compared to peripheral blood [53,57]. Moreover, all blood samples are complex, with fluorescence intensities exhibiting both inter- and intra-donor variability. The variability of the fluorophores is caused by a number of variables: including the donor and the overall heterogeneous nature of blood. Menstrual blood samples have their own level of complexity that can contribute to their inconsistency, involving the variations between cycles and the day of cycle for collection. Understanding how these differences affect the ability to monitor the kinetic changes to bloodstains over time will require further experimentation. As this is an ongoing study, this question will be addressed. Other additional analyses that will be investigated include a longer post-deposition period, increasing the sample size of the donors to incorporate additional races and ages, and determining how different external factors affect the aging. The primary external factors of interest that will be investigated are the potential impact of deposition substrate and the environment during aging, such as varying temperatures, humidity, and sunlight exposure. Once these considerations are examined, a nondestructive and universal technique for the analysis of bloodstains utilizing fluorescence spectroscopy can be created, along with a potential method to determine the time since deposition of bloodstains.

This study was a mirrored experiment to characterizing the biochemical changes that occur within menstrual blood [35]. By measuring the fluorescence of peripheral blood during the aging process, the assignments of the endogenous fluorophores were confirmed, and consistent results were shown for the flavin fluorescence peaks between sample types. By measuring the fluorescence of vaginal fluid at an early time point post deposition, it was revealed that other bodily fluids may present similar fluorescent properties as peripheral and menstrual blood. Therefore, future studies are planned to investigate the fluorescence properties of all forensically relevant body fluids. This will allow a more in-depth look into the biochemical changes that occur within biological samples during drying and aging process. The ability to track and identify time related changes, with further development, can assist in crime scene reconstruction by providing the ability to determine a time window when a crime occurred. As fluorescence analysis is nondestructive, this allows examiners to utilize the analyzed bloodstains for further tests, including DNA profiling. While there is still more research needed, this study is a step forward for utilizing fluorescence spectroscopy's full potential in bloodstain analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2021.112251.

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