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Assessment of *ex vivo* perfused liver health by Raman spectroscopy

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Raman spectroscopy was applied in this research to monitor the overall health and degradation of porcine livers perfused *ex vivo* using the VasoWave® perfusion system. A novel Raman-based diagnostic analysis was developed that enables near real-time and label-free monitoring of organ health during *ex vivo* perfusion designed to extend the useful life of the organ for transplantation. Multivariate statistical analysis of Raman spectra of organ perfusate fluid, using a combination of principal component analysis and linear discriminant analysis, proved to be an effective technique to assess the degradation properties of the livers. Three livers (with replicates) were perfused *ex vivo* under different pressures and temperatures and were compared with a 24-h time-course. Results indicated that perfusion pressure was a more significant factor in organ degradation than was temperature. In addition, a non-linear degradation profile was identified for all three perfused livers, and this profile was different for individual livers, demonstrating the time-dependent transition from its initial 'healthy' state towards a more 'unhealthy' degenerative state at 24 h. The Raman spectroscopy-based approach described here has potential applications in perfusion and diagnostic instrumentation that can be used in near real-time during organ transit and in operating rooms to help identify appropriately healthy organs for transplantation. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: Raman spectroscopy; chemometric analysis; organ perfusion; liver transplantation

Introduction

Liver transplant statistics and challenges

In this research, Raman spectroscopy was investigated as a diagnostic tool to monitor the health of livers awaiting transplantation. Donated organ preservation is needed desperately, as a shortage of transplantable organs exists. As of April 2014, the Organ Procurement and Transplantation Network reported more than 133,000 candidates in the USA alone on the waiting list for an organ (kidney, liver, heart, or lung) and more than 16,000 were on the waiting list for a liver.[1] Chronic liver disease, often caused by cancer, obesity, and/or excessive alcohol consumption, is responsible for more than 16,000 deaths in the USA annually (and about 1.1 million deaths worldwide) and significantly increases health care expenditures.[2–5] Over the years, the list of patients awaiting liver transplantation has increased considerably, because of improved health care that has decreased immediate morbidity/mortality. Unfortunately, a scarcity of donors has also resulted. In the present research, a method of transplantable liver preservation by *ex vivo* perfusion was implemented. The perfusion fluid was monitored in near real-time by Raman spectroscopy, which was found to provide information about liver health. The new methodology was then used to study the effects of *ex vivo* perfusion pressure and temperature by determining the rate of degradation of several livers over a 24-h time-course.

Description of VasoWave® technologies

A new *ex vivo* organ perfusion system, the VasoWave® (Smart Perfusion; Denver, NC), has been developed to preserve organs for transplantation. A unique feature of this system is that it closely mimics physiologic perfusion waveform characteristics of the human circulatory system. Pressurized perfusion fluid is delivered to an organ through the arterial/portal feeds and exits the organ via the venous line providing a closed loop circulation with highly controlled pressures, flow rates, and fluid temperature. The pulse regulator, a patented technology, is the major feature of the system, and it develops and supplies the cardiac waveform associated with the human heart. The VasoWave® system allows the user to instantaneously monitor and program the shape, duration, pulse, and pressure (systolic and diastolic) of a produced waveform. Current hypothermic machine perfusion systems in clinical use do

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not have the ability to real-time monitor parameters such as (i) tissue viability, (ii) metabolic degradation products, (iii) oxygenation state, and (iv) nutrient requirements for an organ undergoing perfusion. The current research addresses this technological gap by combing the novelty of the VasoWave® system with the power of Raman spectroscopy. Most importantly, the use of the VasoWave® system allows lengthy *ex vivo* organ preservation (>12 h) and repetitive sampling of the perfusion fluid bathing the excised organ. This sampling permits temporal measurements of tissue and perfusion fluid analytes that can be correlated with pathophysiology data and other metrics of organ quality. The research data presented here illustrates the use of Raman spectroscopy as a potential near real-time diagnostic tool to monitor total organ health during the perfusion process.

Methods of assessing liver quality

Currently, methods to assess the 'quality' of the donated livers that may be transplanted consist of (i) evaluation of donor characteristics (overall health, co-morbidities, and laboratory tests), (ii) visual inspection of the potentially transplantable organ, and (iii) biopsy/histologic interpretation. Over 50% of potentially transplantable organs are discarded based on these evaluations.[6] More complex tests, such as evaluation of metabolic activity and endogenous quantification of liver function in donors, are costly and impossible within the time constraints of donation/implantation (8–12 h).

Studying the effects of *ex vivo* perfusion on porcine liver health using Raman spectroscopy

The overall goal of this research was to establish a non-invasive diagnostic tool consisting of Raman spectroscopy and multivariate statistical analysis that can provide the first measure of overall *ex vivo* perfused liver health. Raman spectroscopy is gaining momentum in the study of cells, tissues, and organs.[7–11] Major advantages of Raman spectroscopy for analysis of fluid samples include its (i) non-invasiveness, (ii) fast acquisition time, (iii) minimal sample preparation (i.e. no labeling), and (iv) low sensitivity to water. Relatively little research has been performed to date regarding analysis of liver perfusion fluid and tissue by Raman spectroscopy. A previous study using principal component analysis (PCA) of Raman spectra of human liver perfusion fluid found correlation with the oxidation reduction potential of the fluid, which may have ties to organ viability.[12] With biopsied liver tissue, an increase in the intensity of the following Raman bands have been found to be associated with liver injury and fibrosis: 644 cm^{-1} (Tyr), 853 cm^{-1} (Pro, Tyr), 1004 cm^{-1} and 1033 cm^{-1} (Phe), 1083 cm^{-1} (phospholipids), 1303 cm^{-1} (collagen), 1248 cm^{-1} (amide III, collagen, and Tyr), and 1660 cm^{-1} (amide I, collagen, and α-helix).[13] A decrease in Raman band intensity resulting from stretching vibration of the C-S bond of cysteine (660 cm^{-1}) has been associated with fibrosis in biopsied liver tissue. Similarly, liver tissue has been characterized using Raman spectroscopy according to the intensity ratio of phospholipids and collagen (1450 cm^{-1}) to cysteine (1666 cm^{-1}).[13] In the current research, porcine livers were perfused using the VasoWave® system at different operating conditions of temperature and pressure. Raman spectroscopy was applied to study changes in the perfusion fluid in near real-time. Multivariate statistical analysis, specifically PCA followed by linear discriminate analysis (LDA), was applied to Raman spectra to determine the rates and possible mechanisms by which the organs degraded under the different perfusion conditions. This multivariate statistical analysis approach was compared with a more traditional method of Raman spectra analysis in which the intensities of specific Raman bands (corresponding to functional groups of key molecules) are compared along a time-course and among samples. Results revealed a significant advantage of the multivariate approach that makes use of the entire Raman spectra as opposed to specific Raman bands. Thus, the ability to monitor the health of livers in near real-time as they are perfused *ex vivo* may have significant value as donor organs are transported as well as in the operating room immediately prior to transplantation.

Materials and methods

Animals and liver conditioning

Porcine liver procurement methods have been described elsewhere[14] and were used in this research with some modifications. Livers were obtained from humanely sacrificed mixed breed female pigs. Within 15 min of death, livers were harvested, flushed of whole blood with ice cold (4°C) modified Krebs–Henseleit solution, with added heparin anticoagulant, and stored on ice. Livers were stored for 2 h after procurement for transport to the laboratory to begin perfusion with the VasoWave® system, as described in the succeeding section. To facilitate connection with the perfusion system, Luer lock syringe connections were made with the portal vein, hepatic artery, and major hepatic vein. Livers were then perfused with Krebs–Henseleit solution on the VasoWave® system. The Krebs–Henseleit solution consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$ $7H_2O$, 1.2 mM KH_2PO_4, 10 mM glucose, 2 mM $CaCl_2$ $2H_2O$, 4.2 mM $NaHCO_3$, 200 mM sulphinpyrazone, and 10 mM HEPES buffer.

Liver perfusion by VasoWave®

The VasoWave® perfusion system (Smart Perfusion; Denver, NC, USA) was used to perfuse livers *ex vivo* for a period of 24 h under different temperature and pressure profiles. The novelty of this system is its ability to produce a cardio-emulating pulse wave (competing technologies produce a sinusoidal pulse wave) that generates physiological systolic and diastolic pressures and flow rates within a perfused organ. The system also allows for direct control of oxygen content of perfusate. Active perfusion with Krebs–Henseleit solution was initiated on each liver following a 2-h flush. Perfusion was continued for 24 h in a closed circuit. This circuit consisted of the following components: (i) perfusate reservoir bag, (ii) cardiovascular emulation system pump, (iii) heat exchanger, (iv) pressure regulator, (v) atrial line, (vi) perfused liver, (vii) ventricular line, and (viii) recycle stream. Samples of the perfusion fluid were collected at eight time points (0, 0.5, 1, 2, 4, 8, 16, and 24 h) for each liver. A total of six porcine livers were studied in this research, which allowed for three experimental conditions with replicates. The effects of temperature (3°C and 25°C) and pressure (70–30 and 110–70 mmHg) were studied. Specifically, (i) condition A used a perfusion systolic–diastolic pressure of 70–30 mmHg and temperature 3°C, (ii) condition B consisted of 110–70 mmHg pressure and 25°C, and (iii) condition C consisted of 70–30 mmHg pressure and 25°C. Fluid samples were also collected at selected time points from livers maintained only in static cold storage, for comparison with VasoWave® perfusion fluid samples.

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Raman spectroscopy

Ten micro-liters (10 μL) of perfusate fluid was air dried on an aluminum surface at room temperature and analyzed by Raman spectroscopy. Samples were analyzed using a Bruker Senterra dispersive Raman spectrometer (Bruker Optics; Billerica, MA, USA) equipped with a confocal microscope. The sample excitation was performed with a 532 nm laser set to 20 mW power and focused through a 100× objective lens. A scan time of 20 s and a spectral resolution of 9–15 cm^{-1} were used for all measurements. For each sample, a minimum of 50 individual spectra (from different locations of the dried sample) were acquired and used in statistical analyses (discussed in the succeeding section). All spectra were baseline corrected and analyzed using OPUS 7.2 software (Bruker Optics). All spectra were further normalized using methods described in the succeeding section prior to multivariate statistical analyses.

Fatty acids analysis

Extraction of liver perfusate fatty acids was performed following a published protocol[15] with modifications detailed in the succeeding section. Fatty acids from liver perfusates (150 μL) were extracted with 2 volumes of chloroform and 1 volume of methanol in the presence of 10 μg of heptadecanoic acid (absent from liver perfusates) as an internal standard to account for analyte recovery. The chloroform phase was dried under a stream of nitrogen gas, and fatty acids were then esterified to fatty acid methyl esters (FAME) in the presence of 1 N methanolic HCl. FAME were extracted with heptanes and analyzed by gas chromatography coupled with flame ionization detection (GC-FID) after the initial identification of FAME peak identities by mass spectroscopy (MS). FAME separation and analysis was performed using an Agilent 7890A series GC and a 5975C series single quadrupole MS or FID (Agilent Technologies; Santa Clara, CA, USA) equipped with a 30-m DB23 column (0.25 μm × 0.25 mm, Agilent Technologies).[15]

Total protein measurements

The amount of total protein in perfusion fluids was measured (triplicate per sample) using a Coomassie Plus™ (Bradford) Assay kit (Thermo Scientific; Rockford, IL, USA) following the manufacture's protocol. Perfusion fluids were used as is with no further treatment for total protein analysis. Data was acquired by measuring absorbance at 595 nm using a BioTek Synergy H4 Hybrid Multimode Microplate Reader (BioTek; Winooski, VT, USA).

Statistical methods

Statistical analyses were performed to (i) identify correlations between relevant Raman spectral bands and measured fatty acid and protein data, (ii) identify sample outliers, and (iii) separate and cluster Raman spectra according to liver treatment parameters. All calculations were performed using MATLAB (R2012b) (MathWorks; Natick, MA, USA). All spectra were vector normalized over the entire wavenumber range of the spectra. First, correlations between Raman band intensities and experimentally measured FAME and total protein values were found by calculating the correlation coefficient (R) between experimental data points and Raman intensities at several wavenumbers. Multiple Raman band assignments have been made in the literature to different fatty acids and total protein, and this analysis was used to test which of those candidates produced an accurate representation of liver perfusate fluid. The entire list of candidates examined has been published previously.[16] PCA was applied to identify and remove spectral outliers. With the remaining Raman spectra, LDA was applied in order to separate and cluster samples based on (i) the liver analyzed, (ii) temperature, (iii) pressure, and (iv) the time point of sampling. Instead of using the entire spectral range (600–3200 cm^{-1}) for LDA, PCA was first performed to reduce the spectral dimensionality (i.e. the number of Raman intensity data points associated with each spectra). Dataset reduction was found to be important, in cases where LDA was applied to a large number of spectra, in order to reduce computational cost and increase the probability of finding the best model that represents the data (i.e. avoiding overfitting). Spectral dataset reduction using PCA was performed using Raman intensities from the entire spectral region (600–3200 cm^{-1}). The resulting first 50 principal components of a spectrum were used as the reduced dataset and were inputs for LDA analysis. This was based on a previously published finding,[17] and this reference contains an expanded description of the statistical methods involving PCA and LDA.

Results

Reproducibility and changes observed in Raman spectra

Livers were perfused on the VasoWave® system under different pressures and temperatures and were compared (i) with several time points following perfusion onset and (ii) with unperfused livers undergoing cold static storage. For each set of the conditions (i.e. liver, pressure, temperature, and time point), a minimum of 50 Raman spectra of perfusion fluid (or of stagnant fluid of unperfused livers) were obtained, baseline corrected (using OPUS software), and vector normalized (in MATLAB). Averaged Raman spectra for a perfused liver at 0 h, 4 h, and 8 h are shown in Fig. 1(a). Results were found to be highly reproducible, resulting in very low standard deviations [shown in Fig. 1(b)]. The multivariate statistical analysis applied in this research made use of the entire spectra, as opposed to Raman intensities at specific wavenumbers (this is why individual Raman bands are not labeled in Fig. 1). Clear differences in Raman spectra appeared throughout the duration of perfusion. In particular, the overall signal intensity of the biological region of the Raman spectra [Fig. 1(a)] decreased with time; however, noticeable band shifts were observed and certain band intensities decreased faster than others, suggesting these result from metabolic changes of the liver itself. In addition, Raman scans of the initial blood cleared from the liver revealed no similarities with the perfusate scans (results not shown), which suggest the observed effects are not due to clearing of residual blood with time by perfusion. In addition, averaged Raman scans of perfusate sampled from livers perfused under conditions A (70–30 mmHg, 3°C), B (110–70 mmHg, 25°C), and C (70–30 mmHg, 3°C) at 8 h are shown in Fig. S1 (Supporting Information) for the (a) biological region (600–1800 cm^{-1}) and (b) –CH dominated region (2800–3100 cm^{-1}). Thus, perfusate conditions, in addition to time, were observed to significantly impact resulting Raman spectra. While Figs. 1 and S1 (Supporting Information) demonstrate the ability of Raman spectroscopy to detect the subtle changes in response to environmental conditions and duration of perfusion, more meaningful results were not extracted from the individual bands of these datasets. The molecular marker(s) of liver degradation have not yet been identified as distinct Raman bands in Figs. 1 and S1 (Supporting Information). Attempts to correlate individual Raman bands with fatty acids and total protein are

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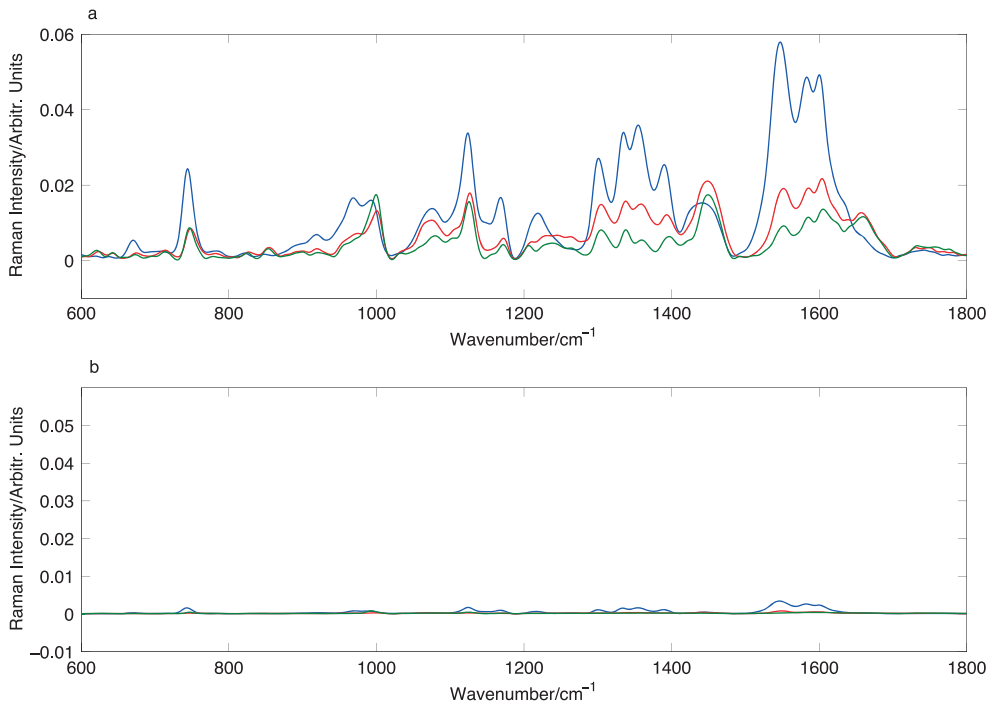

Figure 1. (a) Raman spectra of perfused porcine liver at the following time points: 2 h before perfusion begins (blue), 1 h of perfusion (red), and 8 h of perfusion (green). (b) The standard deviations of 50 replicate scans for each sample.

discussed in a following section. However, to make meaningful conclusions, multivariate analysis involving PCA and LDA with the entire Raman spectra was required. This is demonstrated in the following sections.

Multivariate statistical analysis of Raman spectra

Linear discriminate analysis was performed as described in the methods section on PCA-reduced Raman spectra of sampled fluids from perfused and unperfused livers. Results are shown in Fig. 2(a) for perfused and unperfused samples taken at 0 h and 8 h. LDA produced clear separation among the perfused samples; however, only a small separation was observed for the unperfused samples. The unperfused samples did not undergo the initial flush procedure, so the Raman signal was likely dominated by blood and stagnant fluid that changed in composition very little over the 8 h cold static incubation period. However, this does not imply that the organ did not undergo significant degradation during this time. Having shown that LDA could easily separate perfused and unperfused samples, the next challenge was to establish separation based on perfusion conditions. Three sets of results were generated. The first set [shown in Fig. 2(b–d)] considers three livers perfused under perfusion conditions A, B, and C. The second set (Figs. S2–S4 of Supporting Information) considers the replicate three livers under perfusion conditions A, B, and C. The final set (Figs. S5–S7 of Supporting Information) provides the multivariate statistical results when all six livers are considered simultaneously. The three livers (and replicates) were subjected to different perfusion conditions [A (70–30 mmHg, 3°C), B (110–70 mmHg, 25°C), and C (70–30 mmHg, 25°C)] and were compared across one varied condition at a time. All Raman spectral data for all livers were combined, and LDA was first applied to discriminate according to pressure. Results are shown in Fig. 2(b) for the first three livers tested (without replicates). Clear separation was observed between livers from condition B (perfused at 110–70 mmHg) and conditions A and C (perfused at 70–30 mmHg). This indicates pressure was a significant factor in determining the differences among the reduced Raman spectra of the livers studied. Next, LDA was applied to classify Raman spectra according to temperature of perfusion fluid, and results for the three livers are shown in Fig. 2(c). While separation was still observed, significant overlap of clusters was also present. These results suggest that both pressure and temperature are important perfusion parameters. While the influence of temperature may be more intuitive, results show that changes in pressure directly influence the metabolic activity and degradation kinetics of an *ex vivo* perfused liver. This result has already been established and was the basis for the design of the VasoWave® instrumentation[18]; however, it can now be measured and visualized by Raman spectroscopy and multivariate statistical analysis. Finally, the Raman spectra were classified using LDA on the basis of time (hours of perfusion). Results are shown in Fig. 2(d) (again, for the three livers) and establish a trend showing the overall progression from the initial sample (0 h) to the final sample (24 h). Of course, this trend comprises both pressure and temperature conditions. These results suggest that an LDA model can be used to determine how far a perfused liver has degraded from its initial state. These analyses were repeated for the three liver replicates (Figs. S2–S4 of Supporting Information) and for the set of all six livers considered simultaneously (Figs. S5–S7 of Supporting Information). Results showed a clear influence of both pressure and temperature for the additional three livers (Figs. S2–S3 of Supporting Information), and similar degradation kinetics were revealed compared with the first three livers [Figs. S4 of Supporting Information and 2(d)]. When all six livers were considered simultaneously, the perfusion pressure had a larger influence than temperature (Figs. S5–S6 of Supporting Information); although, this was not seen with the second set of three livers (Figs. S2–S3 of Supporting Information). In addition, the degradation kinetics were

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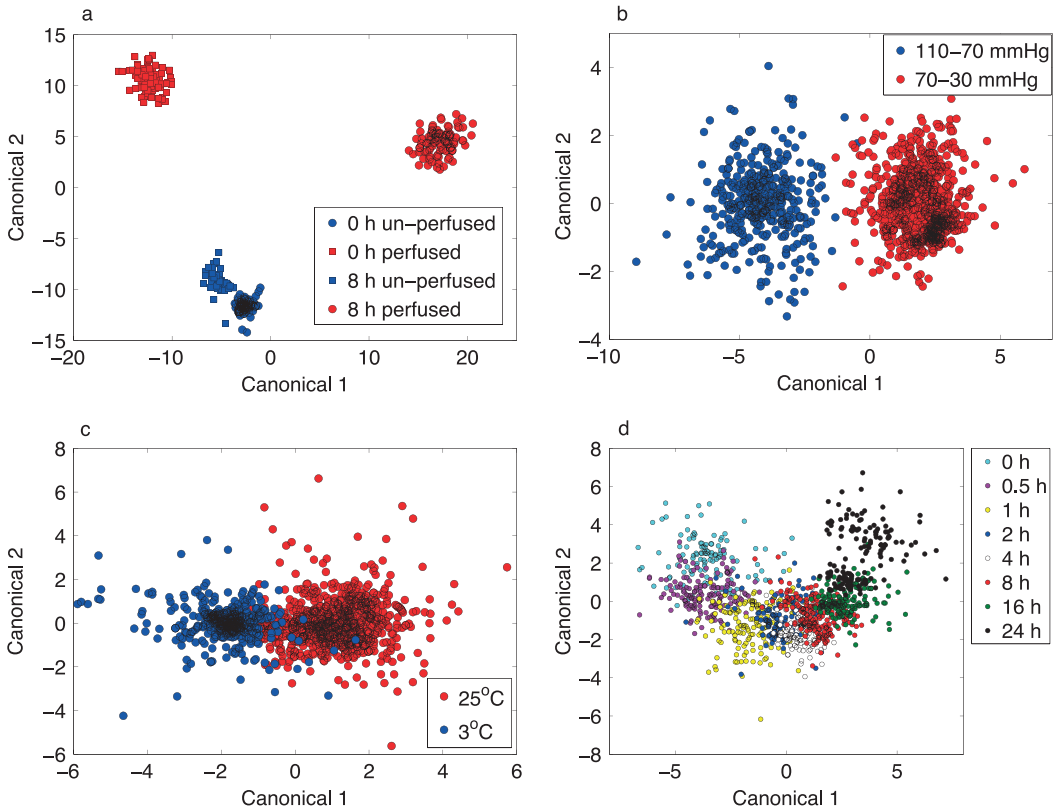

Figure 2. Discriminant analysis results of (a) unperfused and perfused livers at 0 h and 8 h, (b) all data points for livers perfused under conditions A, B, and C discriminated by pressure, (c) all data points for livers perfused under conditions A, B, and C discriminated by temperature, and (d) all data points for livers perfused under conditions A, B, and C discriminated by time.

similar with the livers considered simultaneously as when they were considered individually (Fig. S7 of Supporting Information).

Establishing a measure of time-dependent liver health

The combination of PCA spectral reduction and LDA was performed with respect to time along one dimension [as opposed to the two-dimension analysis of Fig. 2(d)] for the initial three livers exposed to perfusion conditions A (70–30 mmHg, 3°C), B (110–70 mmHg, 25°C), and C (70–30 mmHg, 25°C). The purpose of this analysis was to quantify the rate(s) at which each liver transitioned between its initial and final states. The average of each cluster was calculated along the one-dimensional space, and its distance was calculated from the averaged value from the initial and final time points. This allowed calculation of the 'fraction of degradation', which is defined as the distance of a sample from the initial time point per the distance between the initial and final time points. This provides a time-dependent progression of each liver from its initial state (i.e. 0 h) to its final state (i.e. 24 h). LDA with time, given clear initial and final organ states (i.e. 0 and 24 h), revealed the dynamics of how the liver changed with respect to time. Clear trends were established and significant differences were observed with treatment conditions. The liver from condition A (70–30 mmHg, 3°C) degraded rapidly, with the sample at 2 h nearly mimicking the sample at 16 h, and both were located much closer to the 24 h sample than the 0 h sample [Fig. 3(a)]. In particular, the sample taken at 4 h revealed 76% degradation of the liver from its initial state (0 h) to its final state (24 h). Until proper metrics are established, it can be assumed that 0 h represents a 'healthy' state and the 24 h sample represents an 'unhealthy' liver. The liver from condition B (110–70 mmHg, 25°C) showed slower degradation relative to the liver of condition A (70–30 mmHg, 3°C). The sample from the liver under condition B (110–70 mmHg, 25°C) taken at 4 h revealed 63% degradation from the initial to final state [Fig. 3(b)]. Finally, liver of condition C (70–30 mmHg, 25°C) showed a prolonged period of time where the liver more closely resembled the initial 'healthy' state rather than the final 'unhealthy' state [Fig. 3(c)]. In this case, only 23% degradation was observed at 4 h. However, it must be noted, that this analysis only shows the dynamics of the degradation of a liver from its initial to final state. The final states of the three livers are all different, and their initial states are different as well, because all three livers came from separate animals. In fact, the analyses of Fig. 2(b and c) indicate the final states of the livers perfused with conditions A (70–30 mmHg, 3°C) and C (70–30 mmHg, 25°C) are more closely matched than the livers perfused with conditions B (110–70 mmHg, 25°C) and C (70–30 mmHg, 25°C), despite the fact that the livers with conditions A (70–30 mmHg, 3°C) and C (70–30 mmHg, 25°C) underwent significantly different rates of degradation.

Correlating Raman bands with GC-FID FAME analysis results

Fatty acid methyl esters analysis data were collected for the time-course perfusate samples taken from a representative liver from all three sets of perfusion conditions. Changes were observed for saturated and unsaturated fatty acids content of the perfusate fluid for the three livers analyzed. Results are shown in Fig. 4(a) for saturated fatty acids and Fig. 4(b) for unsaturated fatty acids. The overall saturated and unsaturated fatty acid content remained relatively constant for livers perfused under conditions

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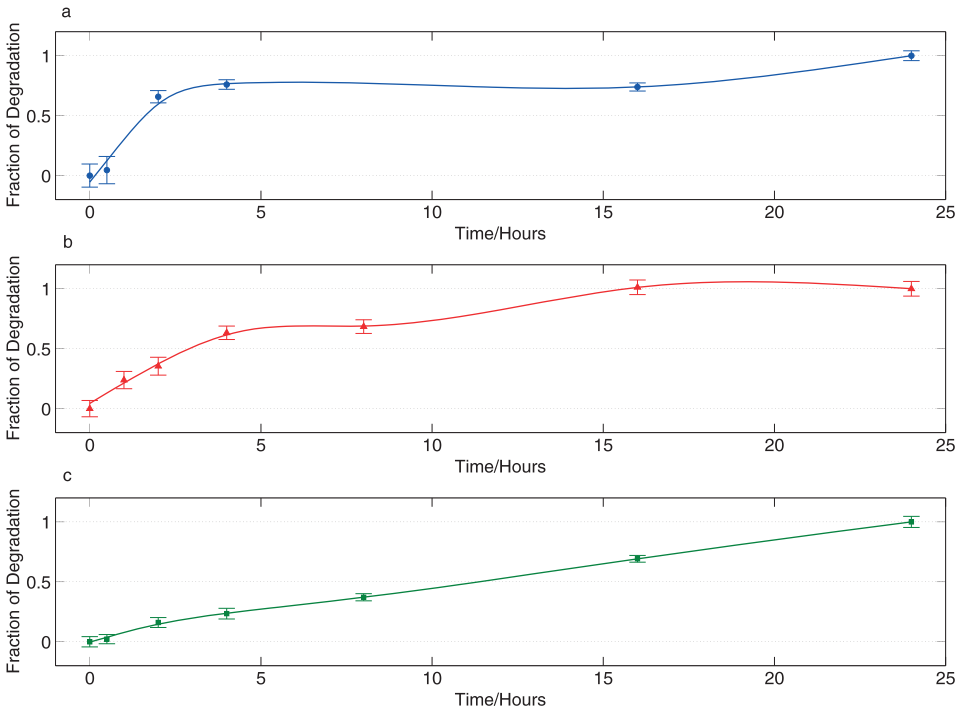

Figure 3. One-dimensional linear discriminant analysis by time for livers perfused under conditions (a) A, (b) B, and (c) C. The initial state of each liver is denoted by the 'Fraction of Degradation' equal to 0, and the final state has a value equal to 1.

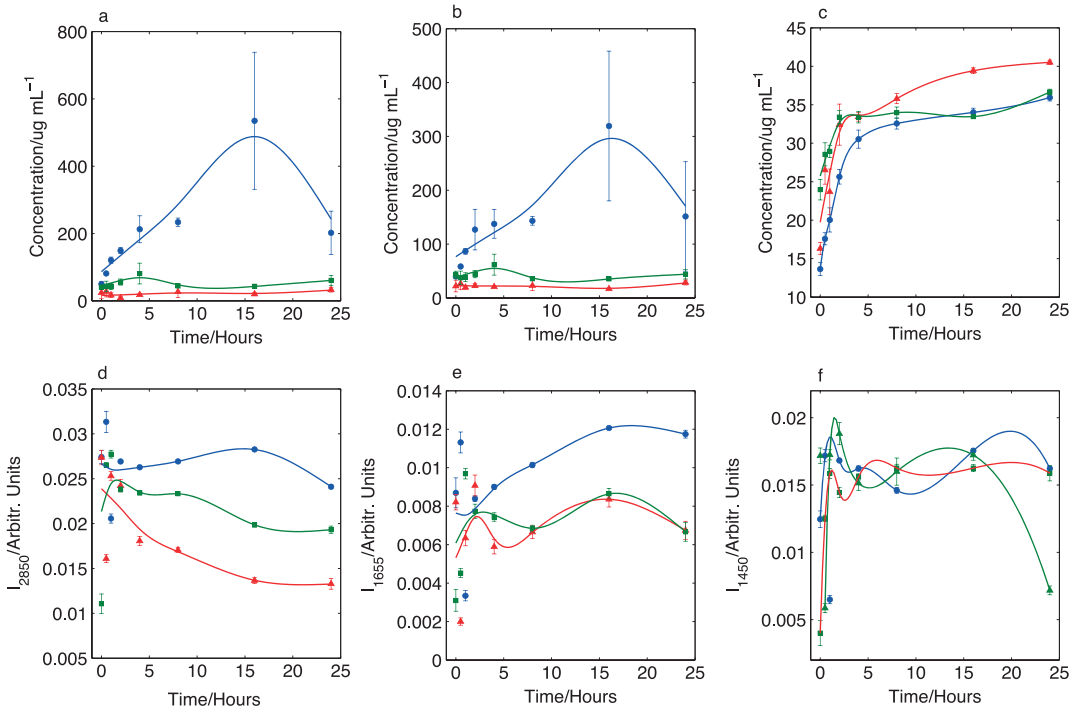

Figure 4. (a) Saturated fatty acids measured by gas chromatography coupled with flame ionization detection (GC-FID), (b) unsaturated fatty acids measured by GC-FID, (c) experimental measurements of total protein, (d) saturated fatty acids measured by Raman spectroscopy using peak intensity at 2850 cm^{-1}, (e) unsaturated fatty acids measured by Raman spectroscopy using peak intensity at 1655 cm^{-1}, (f) total protein measured by Raman spectroscopy using peak intensity at 1450 cm^{-1}. Liver perfused under conditions A (blue), B (red), and C (green) are represented in each plot.

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B (110–70 mmHg, 25°C) and C (70–30 mmHg, 25°C). The liver perfused with condition A (70–30 mmHg, 3°C) exhibited a higher fatty acid content, and 3–4 fold increases were observed over the time-course. This is also consistent with its elevated rate of degradation [Fig. 3(a)]. Correlations of these data with Raman spectra were sought. Similar comparisons were performed for bacteria and high correlations ($R > 0.85$) were identified between Raman and GC-FID data.[16] In the case of perfusate fluid, the Raman band intensities showing correlation after 4 h of perfusion were (i) 2850 cm^{-1} (indicative of CH_2 symmetric stretch of lipids[19]) for saturated fatty acids [Fig. 4(d)] and (ii) 1655 cm^{-1} (indicative of C=C bonds[19]) for unsaturated fatty acids [Fig. 4(e)]. While perfect correlations were not obtained (especially before 4 h), general trends were captured after 4 h of perfusion, and the liver perfused with condition A (70–30 mmHg, 3°C) was shown by both FAME analysis and Raman band analysis to have higher amounts of both saturated and unsaturated fatty acids in the perfusates. However, the Raman band analysis failed to effectively capture trends prior to 4 h of perfusion and overall correlation coefficients between FAME and Raman data were low ($R < 0.5$). However, Raman spectroscopy shows promise for tracking the presence of saturated and unsaturated fatty acids in liver perfusate after 4 h in near real-time.

Correlating Raman bands with total protein measurements

In addition to fatty acids, protein is another major constituent of liver tissue that was expected to accumulate in the perfusion fluid with organ degradation. A comparison of the total protein content of perfusion fluid from livers of each set of perfusion conditions may provide further indication of their physical states. Livers exposed to condition B (110–70 mmHg, 25°C) were perfused at higher pressure and temperature and showed slightly higher protein content in the perfusion fluid; however, livers from all three sets showed increases in perfusate protein content of ~50% over the course of 24 h [Fig. 4(c)]. Raman signatures were also sought with good correlation to total protein measurements. Results are shown in Fig. 4(f) for the Raman band intensity at 1450 cm^{-1}, which has been associated with C-H deformation in amino acid side chains.[19] Similar to the results for fatty acids, good overall trends were observed between protein content measured using standard experimental producer and Raman spectroscopy, but low correlation coefficients were observed (average $R = 0.32$). These results suggest that time-course Raman band analysis can provide only a low confidence estimate of total protein in liver perfusate. However, this measurement can be obtained in near real-time, which is significant for time-sensitive applications such as organ transplantation. Analyses for specific protein markers indicative of liver failure may yield better correlation with Raman data, but these have not yet been investigated.

Discussion

Raman spectroscopy as a diagnostic tool for near real-time analysis of cells, tissues, and organs has many potential applications. Here, Raman spectroscopy was applied to monitor porcine liver health during *ex vivo* perfusion using the VasoWave® system. Clear distinctions were observed between perfused and unperfused organs, and significant changes were detected in the perfusate based on perfusion conditions (i.e. pressure and temperature) and over the 24 h perfusion time-course. It is suspected that the Raman spectra of the unperfused samples remained dominated by stagnant blood; whereas, perfused livers were quickly cleared of residual blood and debris and Raman spectra of perfusate were likely characterized by metabolic byproducts of organ degradation. We have noted similar results in cardiac tissue and kidney perfusate samples (unpublished results). Donor livers preserved for transplantation are known to have a limited lifetime that is on the order of hours. Continuous real-time monitoring of overall organ health is critical because often the transplantation window does not allow for comprehensive off-line analytical analyses to determine if an organ is a good candidate for transplantation. In addition, real-time diagnostics have the potential to allow for continuous monitoring while the organ is in transit as well as in the operating room. The dynamics of organ degradation over the first few hours of *ex vivo* perfusion may very well provide critical information regarding the long-term viability of the transplanted organ and patient. The Raman-based diagnostic tool presented here provides 'near' real-time analysis. The time delay is on the order of seconds (<240 s) and consists of (i) drying time for a 10 μL spot sample, (ii) Raman scanning of several points of the sample, and (iii) computations. It is anticipated that Raman technologies capable of repeatable liquid phase analysis will further reduce this time delay, but liquid measurements led to inconsistent results in this research (data not shown). The use of a 532 nm laser was also required to produce reliable results. Great care must be taken when operating at higher energies due to the risk of graphitization.[20] This was managed by (i) using a short integration time (10 s), (ii) scanning different areas of the sample, (iii) averaging 50 independent scans per sample, and (iv) looking for a wide graphitization band around 1500 cm^{-1} in the Raman spectra.

In this research, a perfused porcine liver was compared with a non-perfused liver, and six livers were subjected to three sets of perfusion pressures and temperatures and monitored over the time-course of *ex vivo* perfusion using the VasoWave®. While significant differences were detected and trends were repeatable in replicates, the sample size is too small to make conclusive statements regarding the effects of perfusion conditions. However, the purpose of this study was to develop and demonstrate Raman spectroscopy with multivariate statistical analysis as a potential near real-time diagnostic tool for monitoring organ health by analyzing perfusate. Because inherent variability exists in different livers, it is likely that individual liver characteristics played a role in its rate of degradation, along with the varied perfusion parameters of the VasoWave®. Large-scale studies are underway and will be used along with clinical diagnostics to distinguish between 'healthy' and 'unhealthy' organs for transplantation.

The use of Raman spectroscopy as a near real-time diagnostic tool requires significant raw data processing and statistical analysis. In the case of complex biological fluids, as observed in liver degradation during *ex vivo* perfusion, we argue for the use of a multivariate statistical analysis approach that makes use of entire Raman spectra rather than comparing intensities of individual bands. Individual band analysis assumes no interference from molecules with similar chemical structures and functional groups. This is ideal for closed systems where all possibilities are known; however, the chemistry of a diseased and/or degrading liver can be extremely complex. Raman bands identified for fatty acids, and protein in this research must be further verified in large-scale studies and may ultimately yield less useful information than a holistic multivariate analysis

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because this approach takes into account all molecular signatures that are changing among all Raman spectra. Thus, it is anticipated that the multivariate statistical analysis approach will best characterize a changing complex system. Regardless of whether Raman band analysis for fatty acids or multivariate statistical analysis proves more beneficial in large-scale studies, both may ultimately have use in time-sensitive applications, such as in organ transplantation. As shown with the Raman band correlations with fatty acid and protein data, sacrificing accuracy for near real-time information is required, but this trade-off may be worthwhile. Furthermore, it is reasonable to speculate that further advances in Raman spectroscopy instrumentation, measurement techniques, and data analysis methods will enhance the accuracy of near real-time measurements.

Even though Raman spectroscopy proved valuable in monitoring the differentiation of porcine liver perfusate fluid in near real-time, challenges still remain to definitively link these changes with organ health. However, the ability to monitor changes in the complex chemistry of a dynamic system, such as a degrading liver, is novel and has significant future medical applications. As additional porcine and human livers are analyzed, a database will be constructed linking Raman signatures to the clinical pathophysiology of these organs. With this information, additional multivariate statistical measures and artificial intelligence models will enable the linking of the changes in Raman spectra to true measures of organ health. This is critical near real-time information that will ultimately be used by surgeons and clinicians when making the critical decision of whether an organ is sufficiently healthy for transplantation into a recipient patient.

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