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Longitudinal analysis of molecular alteration in serum samples of dextran sodium sulfate-induced colitis mice by using infrared spectroscopy

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ABSTRACT

Established diagnostic regimens for inflammatory bowel diseases, such as colonoscopy, sigmoidoscopy, and small bowel follow-through have high sensitivity, but adherence rates of the eligible population for the disease screening is poor due to associated expenses, the risk of complications, time, and comfort issues. Thus, it is critical to develop an **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid, **E**quipment-free, and **D**eliverable (ASSURED) screening test that could increase the participation rate of the eligible group. In this study, we present infrared spectroscopy of serum accompanied with data analysis to monitor longitudinal analysis of molecular alteration in colitis mouse samples. Dextran Sodium Sulfate-induced colitis mouse model sera were used to understand the disease aggravation by testing of the alteration of carbohydrates and the protein secondary structures reflected in the spectra. Samples derived from mouse models at days 0, 3, and 7 of chemical administration were used for spectral measurements. Spectral data at Biomarker peaks show there is not any specific alteration of serum sample composition at day 3 of the chemical feeding, but a significant alteration on the marker can be seen at day 7. The results of this study will thus pave the way for the development of an ASSURED prototype that can have clinical application.

1. Introduction

Inflammatory Bowel Diseases (IBDs) are debilitating, and in the absence of early detection, they can lead to life-threatening complications, including colorectal cancers [1]. Current methods of diagnosis regarding IBDs [2] include colonoscopy, sigmoidoscopy, and small bowel follow through, all of which are invasive, expensive, have associated risks, and can cause great discomfort to patients. These complications result in a decreasing adherence rate of the eligible population for IBD screenings. At present, millions of people within the United States are suffering from these diseases [3] and the number increases annually. Therefore, it is necessary to develop an assured screening regimen for IBDs. Recent studies have reported [4–7] that the infrared (IR) spectroscopy of serum samples with an appropriate data handling framework can be used for rapid and reliable screening of experimental colitis conditions. Spectral-markers representing mannose and protein secondary structures within the sample medium were shown to change due to colitis progression and then return to their base level after the treatment [5]. Although a significant body of knowledge regarding the identification of a unique spectral marker for colitis a major type of IBD- has been accumulated, biomolecular structural alteration and its role in the daily progression of colitis is still not completely understood. This understanding is always critical while developing a diagnostic regimen.

In this paper, we report the longitudinal analysis of molecular alteration in the serum samples of mice, with **D**extran **S**odium **S**ulfate (DSS) induced colitis by using Fourier transform infrared (FTIR) spectroscopy in attenuated total reflectance (ATR) sampling mode. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) [8] spectroscopy is a rapid and reliable analytical technique which can be used to extract snapshots of the morphology and composition of Biomolecules within the softer types of diagnostic mediums [9,10]. Furthermore, the DSS mouse model provides us an inexpensive, simple and reproducible model to study mucin in barrier integrity, alteration in microbial balance and changes in the metabolome of human IBD [11]. Importantly, human IBD is a chronic intestinal inflammation disorder with an unknown etiology. It has been found that genetic and epigenetic factors also play an important role in the onset of disease and its progression [1]. In our earlier works, we have also used genetically modified IL10-/- mouse models of chronic colitis along with other types of mouse models for intestinal as well as extra-intestinal inflammation. Spectral markers presented in this draft are common to both DSS and

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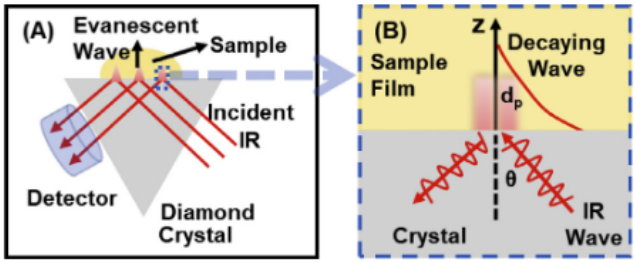

Fig. 1. (A) Schematic diagram of the evanescent wave formed on the sample at the internal reflection element surface. (B) Interaction of infrared signal in the material of the medium.

IL10-/- mouse. In these earlier works, we have analyzed quantified information of Lipocalin 2 (Lcn-2) levels in feces of IL10-/- mice and Myeloperoxidase (MPO) activity of distal colon in DSS induced colitis to monitor disease progression. MPO and human counter part of Lcn-2; neutrophil gelatinase-associated lipocalin (NGAL) alteration has been reported in human studies. This novel diagnostic strategy will generate interest in the scientific community working in the same area.

In ATR-FTIR spectroscopy, light is totally reflected (Fig. 1A) inside the prism of the high refractive index [12], and the reflected light from the sample (Goos-Hanchen effect) [13] creates an evanescent wave, penetrating the sample placed in contact with crystal [14]. The energy of the evanescent wave first interacts with the sample medium, and the incident wave is entirely reflected back into the system as shown in Fig. 1(A). The output spectra represent a snapshot of molecular components within the sample medium. Herein, the penetration depth (Fig. 1B) of evanescent energy waves, $d_p = \lambda/2\pi(n_1{}^2 Sin^2\theta - n_2{}^2)^{1/2}$ depends on the wavelength of incident radiation (λ), the refractive index of the crystal (n_1), the angle of incidence (θ), and the refractive index of the sample (n_2). It is noted that the evanescent wave decays exponentially in relation to the distance from the interface [9], so the penetration depth is only a fraction of incident IR wavelength. The evanescent waves lose energy at frequencies identical to the sample's absorption because soft biological materials [15], such as the serum, make intimate contact with the ATR element.

Blood serum, which includes proteins, electrolytes, antibodies, antigens, hormones and exogenous substance, can also make an intimate contact with the ATR element, i.e. the diamond crystal in our case. ATR-FTIR spectra measurement offers better sample to sample reproducibility, while significantly minimizing the chances of user to user spectral variations when compared to other vibrational analytical techniques for analyzing soft diagnostics mediums [16]. Thus, monitoring longitudinal analysis of molecular alteration in the serum samples is a crucial step in anticipating the development of an ASSURED portable prototype which offers facilities for bedside technology offering a rapid and reliable screening of IBDs.

2. Materials and methods

The animal growth and sample preparation: Six C57BL/6 WT mice were administered drinking water containing 3% DSS and were also fed ad libitum for 7 days by our collaborators. Blood was collected from these mice, at day 0 (before DSS treatment), day 3 and day 7 of DSS chemical feeding. Serum was obtained by centrifugation of the collected blood by using serum separator tubes. All studies were performed in accordance with the Institutional Animal Care and Use Committee at Georgia State University (Atlanta, GA), permit number: A17044.

Equipment setup and sample scanning: A Bruker Vertex 70 FTIR spectrometer series with a KBr beam splitter, and a Deuterated Tri-Glycine Sulfate (DTGS) pyroelectric detector were used. The MVP-Pro ATR accessory from Harrick-Scientific with a diamond crystal (1 mm × 1.5 mm) as the internal reflection element, was configured to have a single reflection, and used to obtain ATR absorbance spectra. Measurements were taken at resolution 4 cm^{-1} and the IR light beam intensity was controlled by passing it through an adjustable aperture set to 2.5 mm.

Background measurements were performed prior to each spectral measurement. Serum samples, one micro-liter were deposited on the crystal surface were allowed to air dry at room temperature (~8min) and were scanned multiple times. It is noted that, when the sample is stable on the crystal surface, obtained spectra are overlapping with their previous spectra. The sample to sample reproducibility and user to user stabilization was also verified in our study. In our measurements, three reads of 100 co-added scans for each sample (total of 300 scans) were averaged to get final spectral for analysis. To maintain the uniformity in each measurement all samples were measured in one sitting (Each measurement takes ~22 min.). The proposed portable setup accompanied with data analysis facility will not increase the chances of its access for clinicians, but also reduces the time for spectral analysis.

***Spectral analysis*:** All the spectra were max-min normalized (OPUS 7.2 software) by scaling the range 1800–900 cm^{-1} so that the absorbance value at 1642 cm^{-1} was 2. Our analysis includes quantification of absorbance values at different biomarker's positions, which follows discrimination of secondary structures by spectral deconvolution at amide I (1600–1700 cm^{-1}), where changes are not obvious from visual inspection. During spectral deconvolution, confined spectra of the selected region were first base line corrected, followed by determination of second derivative spectra. These spectra were then fitted with Gaussian band profiles by approximating the position and number of bands from the second derivative spectra. In the fitting process position, height, width and hence integral are allowed to vary such that simulated curve best fits to the amide I region of the experimental curve. Detail of spectral deconvolution of amide I region, to analyze protein secondary structure is described elsewhere [5,17]. Statistical analysis was done by using quantified absorbance values at different Biomarker positions and integral area of Gaussian function energy bands obtained after spectral deconvolution and are representing protein secondary structure.

3. Result and discussion

To monitor the temporal variation of molecular composition in the serum along with the colitis aggravation, we examined samples at days 0, 3, and 7 of the chemical feeding. Six mice (S1, S2, S3, S4, S5, and S6) were fed 3% DSS dissolved in drinking water. The serum samples were tested for the disease progression using the spectral response analysis. After analyzing the absorbance spectra within spectral range 1000–1140 cm^{-1}, we learned the concentration of carbohydrates such as glucose and mannoses were elevated due to colitis as judged in previous studies [4–7]. These studies have shown that the elevation of the spectral band representing the mannose presence in the carbohydrate region [4], increased the presence of β-pleated sheet structures and decreased the presence of α-helical structures [5] in the amide I region (1600–1700 cm^{-1}).

Fig. 2(A) shows the average (n = 6) of max-min normalized spectra of mice sera extracted from three different time periods before (days 0, 3, and 7 of chemical feedings) the DSS mouse model, with a Biomolecular assignment at different wavenumber regions. Our study is primarily focused on testing the temporal variation in serum components along with the colitis progression by analyzing identified unique spectral markers of colitis (shaded regions in Fig. 2). The second derivative curve of the absorbance spectra is shown in Fig. 2(B). Visual inspection of each type of spectrum shows alteration of disease Biomarkers due to colitis. This variation can be seen at day 7 of DSS feeding, but not at day 3. It is noted that the deviation of repeat measurements within the control or colitis data in identifying spectral peaks is negligible compared to disease associated deviations (not shown in the figure). The sample to sample reproducibility and the user to user stabilization was also verified in our study. Sample to sample reproducibility and user to

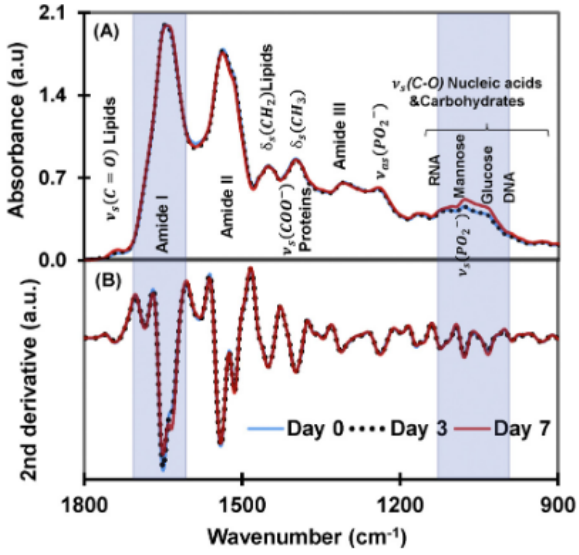

Fig. 2. (A) Averaged (n = 6) ATR-FTIR spectra of serum sample derived from DSS mice at day 0, day 3 and day 7 of the chemical feeding. (B) Secondary curves of the absorbance spectra. Herein, shaded regions show two major regions of interest.

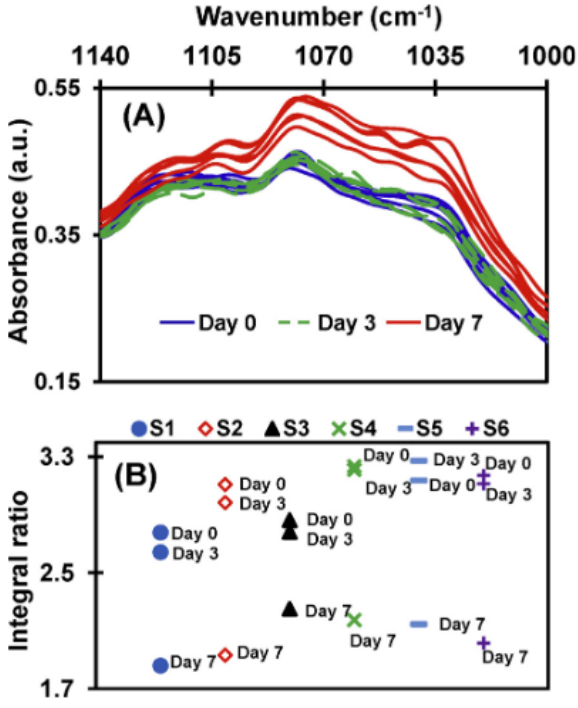

Fig. 3. Longitudinal analysis of serum samples of DSS induced colitis mice (A) Normalized absorbance values in the range 1000–1140 cm^{-1}. (B) The integral ratio of Gaussian function energy bands representing α-helix and β-sheet secondary structures of proteins in serum samples extracted from six mice.

user stabilization are crucial features for any diagnostic regimen. Using eight repeat measurements from a random sample, the absorbance value of the mannose peak of normalized data is 0.44 (± 0.01) for the control and 0.49 ((± 0.02) for colitis; a similar data trend in other spectral peaks was obtained. There is a negligible variation between these spectra compared to changes induced by colitis, therefore proving the feasibility of this approach in clinical chemistry tests. Fig. 3(A) shows the normalized absorbance spectra covering the carbohydrate region. The ratio of the integral area taken by Gaussian function energy bands representing α-helical and β-pleated protein secondary structures

Table 1
Biomolecular assignments of major infrared spectral bands unique to colitis. The statistical significance of colitis-associated alteration in these spectral markers is also highlighted.

	Spectral region (cm^{-1})	Biomolecular assignment vibrations	p-value comparison	Explanation (Difference)
Carbohydrate (1000–1140)	1020	Glucose/DNA	2E-04	Yes
	1033	Glucose	2E-04	Yes
	1060	Fructose	8E-07	Yes
	1076	Mannose	1E-05	Yes
	1108	Endocyclic C–O–C vibration	6E-04	Yes
	1122	DNA, RNA, Phospholipids	0.62	No
Amide I (1600–1700)	1610	Side chain	0.86	No
	1630	β pleated sheet	6E-03	Yes
	1648	Random coil	0.82	No
	1652	α-helix	1E-03	Yes
	1682	β (anti-‖ + turn)	0.78	No

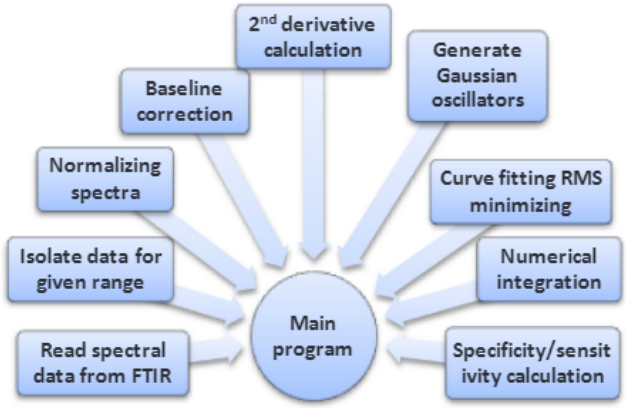

Fig. 4. Structure of the software program showing 9 subroutines to perform each specific task. Each of these specific programming tasks was individually carried out in our preliminary work in order to get the final conclusion. In the portable device development, all those will be automated into one program which will be done at a single touch command.

for each individual mouse is shown in Fig. 3(B). Analysis of absorbance at the Biomarker peaks of spectra at day 3 of DSS administration show no specific changes, but at day 7, a statistically significant alteration on marker position can be seen.

In the normalized data, absorbance values at identified unique spectral position, representing mannose, 1076 cm^{-1} for the control (day 0) condition are between 0.44 and 0.47 a.u., colitis (day 7) is 0.49–0.53 a.u., and that of intermediate (day 3) stage lies within the range of control. The integral ratio of α-helix and β-sheet protein structures also show a clear difference in the spectral data representing day 0 with those representing day 7 of chemical administration, while there is no significant difference in day 3 data. Biomolecular assignments of major components in the regions of interest are tabulated as follows. At the colitis stage (day 7), spectral bands associated with Glucose, Fructose, Mannose, and Endocyclic C–O–C vibration, α-helical structure of amide I, β pleated sheet structures of amide I show alteration with higher statistical significance (student's *t*-test, two-tailed unequal variance p-value < 0.05), while others remain identical in control as tabulated below (see Table 1).

4. A prototype of the portable device

Our study is also directed toward designing and developing a prototype of a portable tool for facilitating such measurements. As the first

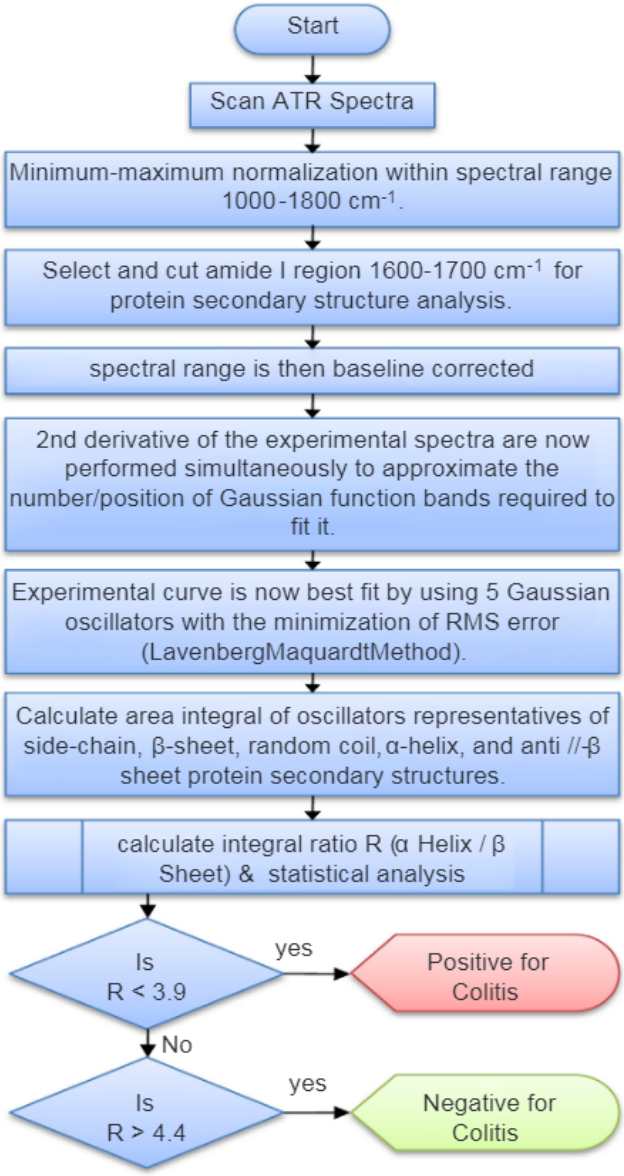

Fig. 5. Flow chart for the programming to study protein secondary structures. Only the α-helix and β-sheet ratio analysis is shown in the flow chart. Similarly, other biomarker analysis will also be in the program as needed to achieve the needed confidence limit.

stage of the portable device development, we have studied the possible use of commercially available small FTIR (Brucker ALPHA & Perkin Elmer 2) with single or multi-reflection ATR setup. The communication with the commercial software from the FTIR manufacturer will be critical to develop a user friendly prototype, so accompanying software can be developed to completely automate the process. Fully automating the spectral measurements and analysis presented above is the second step to check the feasibility of the proposed portable device. Minimization of the instrumentation by integrating it with a simple touchscreen (e.g. Windows tablet) will make it a user friendly desktop unit. Spectral measurements and data analysis procedure will be automated into a single step so that a technician can deposit the sample onto the sample holder and start the measurement with a simple click to get the result and if needed the biochemical information.

The software program will include 9 subroutines as shown in Fig. 4 and each will do a specific task in the data extraction or analyzing stage. Reading a spectral data file from the FTIR, extract data for a suitable biomarker range depending on the user-selected disease,

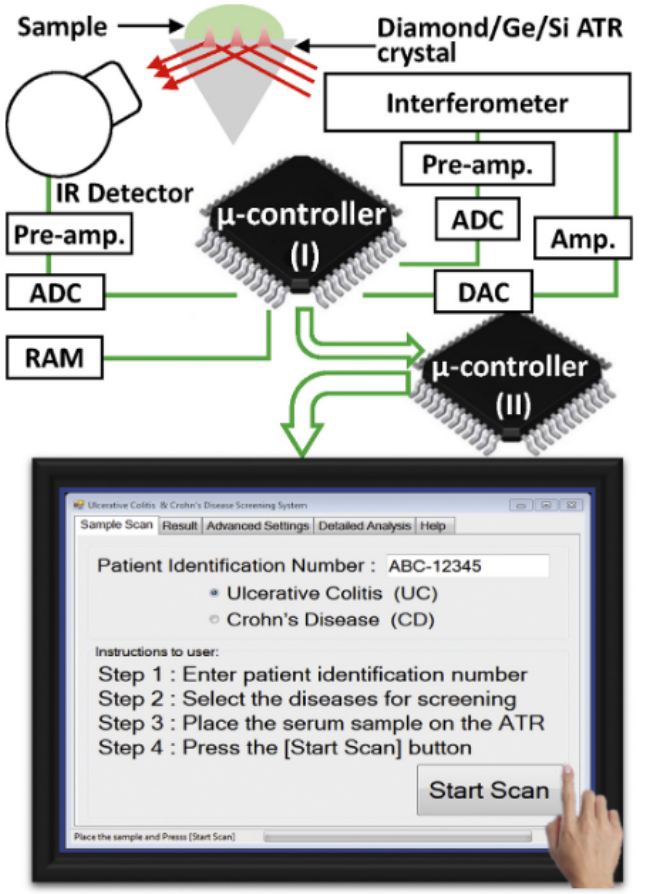

Fig. 6. Schematic of measurements and data analysis tool permanently integrated into the anticipated modified spectroscope. "Sample Scan" tab allows users to enter patient ID, select the disease followed by pressing "Start Scan" to initiate the program.

Fig. 7. Anticipated portable device with the touch screen showing "Test Report". The test report can be printed as a hard copy or save the patient record in the hospital database.

normalizing and baseline correction of spectral data subroutines will have simple loops, condition check, and basics mathematical calculations. The second derivative will be calculated by using divided difference formulas for discrete data. After finding the number of minimums and their positions, the program will assign parameters for Gaussian oscillators. The curve fitting subroutine will iteratively vary the Gaussian curve parameters to minimize the RMS error between experimental absorption curve and the corresponding summation of the Gaussian curves. The Levenberg Marquardt algorithm will be used in this subroutine. The standard numerical integration technique will be used to find the area under each Gaussian oscillator. As an example, the flow chart for protein secondary structure analysis is shown in Fig. 5. Other biomarkers already identified and new ones to be identified in the process can also be included in the program. For debugging and troubleshooting reasons, it is important to be able to see the output at each stage in addition to the final outcome. Also, this allows anyone interested to see the important data at each stage of the process and to try other changes.

A schematic of this anticipated portable prototype for the colitis screening by using ATR-FTIR spectroscopy is shown in Fig. 6, where the microprocessor (I) controls all the functions in the instrument, and the software (spectral measurement and data analysis) will run on a microprocessor (II). The software package developed can be installed on the Windows tablet PC attached to the portable FTIR. Herein, the "Sample Scan" tab allows users to enter patient ID, select the disease, followed by the user pressing "Start Scan" to initiate the program. Pressing "Start Scan" after depositing a sample on the holder can automatically perform the full data analysis and will display laboratory test report as seen in Fig. 7.

5. Conclusion

This study demonstrates the application of the IR spectroscopic technique for monitoring colitis aggravation on days 0, 3, and 7 of chemical administration on DSS mouse models of colitis. It also reveals as a preliminary step in anticipating the validity of the test for humans and the development of a portable prototype which can facilitate the bedside IBD screening. Monitoring alteration in the unique spectral markers such as concentration of carbohydrates (glucose and mannose) and the alpha helix to beta sheet ratio of the protein secondary structure reflected in the IR spectra of serum, for colitis during disease progression and suppression will allow us to understand the feasibility of the presented diagnostic regimen in the clinical domain. Also, the DSS mouse started to behave differently at day 3 of the chemical feeding, so present study using samples on days 0, 3, and 7 of chemical administration, is the foundation for further research with large sample size. Further work is in progress to investigate dose-dependent aggravation, and disease progression of intermediate stages at day five and its suppression after twelve days, after stopping the chemical feeding. Herein, the DSS mouse model eliminates few challenges of the human studies and allows us to conduct disease research on the controlled environment, but there are several other challenging aspects to translate these findings to humanistic studies. The change in spectral markers along with disease progression and suppression needs to be confirmed with the use of sample of the human patients. Thus, in our future work, spectral markers will be further confirmed by using serum samples from colitis patients. Once the unique spectral markers are identified and confirmed, an ASSURED portable prototype can be developed in which measurements and data analysis techniques can be automated in the software, so that a technician will deposit the specimen onto the sample holder and start the measurement with a simple click to get the result. This rapid, simple, cost-effective, and minimally invasive technique would allow the assessment of disease status and personalized drug management in the future health care of IBD patients.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.infrared.2018.11.034.

References

[1] S.H. Itzkowitz, X. Yio, Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation, Am. J. Physiol.-Gastrointest. Liver Physiol. 287 (2004) G7–G17.
[2] S. Vermeire, G. Van Assche, P. Rutgeerts, Laboratory markers in IBD: useful, magic, or unnecessary toys? Gut 55 (2006) 426–431.
[3] R.J. Colman, D.T. Rubin, Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis, J. Crohn's Colitis 8 (2014) 1569–1581.
[4] J. Titus, E. Viennois, D. Merlin, A. Unil Perera, Minimally invasive screening for colitis using attenuated total internal reflectance fourier transform infrared spectroscopy, J. Biophoton. 10 (2017) 465–472.
[5] J. Titus, H. Ghimire, E. Viennois, D. Merlin, A. Unil Perera, Protein secondary structure analysis of dried blood serum using infrared spectroscopy to identify markers for colitis screening, J. Biophoton. 11 (2018) e201700057.
[6] J. Titus, H. Ghimire, E. Viennois, D. Merlin, A.U. Perera, Infrared spectroscopy as a screening technique for colitis, Bio-MEMS and Medical Microdevices III, 2017, p. 102470C.
[7] A.U. Perera, H. Ghimire, K. Perera, E. Viennois, D. Merlin, Colitis screening using IR spectroscopy of serum samples, Sensing Technology (ICST), 2017 Eleventh International Conference on, 2017, pp. 1–4.
[8] S. Kazarian, K. Chan, Applications of ATR-FTIR spectroscopic imaging to biomedical samples, Biochim. Biophys. Acta (BBA)-Biomembranes 1758 (2006) 858–867.
[9] K.A. Chan, S.G. Kazarian, Attenuated total reflection Fourier-transform infrared (ATR-FTIR) imaging of tissues and live cells, Chem. Soc. Rev. 45 (2016) 1850–1864.
[10] H. Ghimire, M. Venkataramani, Z. Bian, Y. Liu, A.U. Perera, ATR-FTIR spectral discrimination between normal and tumorous mouse models of lymphoma and melanoma from serum samples, Sci. Rep. 7 (2017) 16993.
[11] D.D. Eichele, K.K. Kharbanda, Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis, World J. Gastroenterol. 23 (2017) 6016.
[12] S.G. Kazarian, K.A. Chan, ATR-FTIR spectroscopic imaging: recent advances and applications to biological systems, Analyst 138 (2013) 1940–1951.
[13] A.W. Snyder, J.D. Love, Goos-Hänchen shift, Appl. Opt. 15 (1976) 236–238.
[14] G. Clemens, J.R. Hands, K.M. Dorling, M.J. Baker, Vibrational spectroscopic methods for cytology and cellular research, Analyst 139 (2014) 4411–4444.
[15] M.J. Baker, S.R. Hussain, L. Lovergne, V. Untereiner, C. Hughes, R.A. Lukaszewski, et al., Developing and understanding biofluid vibrational spectroscopy: a critical review, Chem. Soc. Rev. 45 (2016) 1803–1818.
[16] K.M. Dorling, M.J. Baker, Highlighting attenuated total reflection Fourier transform infrared spectroscopy for rapid serum analysis, Trends Biotechnol. 31 (2013) 327–328.
[17] H. Yang, S. Yang, J. Kong, A. Dong, S. Yu, Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy, Nat. Protoc. 10 (2015) 382.