Published in final edited form as:

J Raman Spectrosc. 2013 August 1; 44(8): 1089–1095. doi:10.1002/jrs.4338.

Laser Wavelength Dependence of Background Fluorescence in Raman Spectroscopic Analysis of Synovial Fluid from Symptomatic Joints

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Abstract

Gout is a disease process where the nucleation and growth of crystals in the synovial fluid of joints elicit painful arthritis-like symptoms. Raman spectroscopy is evolving as a potential diagnostic tool in identifying such crystals; however, attainment of sufficient Raman signal while overcoming the background fluorescence remains as a major challenge. The current study focused on assessing whether excitation in 532–700 nm range will provide greater signal intensity than the standard 785 nm while not being impeded by background fluorescence. We characterized the fluorescence spectra, absorption spectra and Raman spectra of synovial fluid from patients who presented “gout-like symptoms” (symptomatic) and controls (asymptomatic). A digestion and filtration method was developed to isolate crystals from synovial fluid while reducing the organic burden. Spectral profile and photobleaching dynamics during Raman spectroscopy were observed under an excitation wavelength range spanning 532 to 785 nm. Absorbance and fluorescence profiles indicated the digestion and filtration worked effectively to extract crystals from symptomatic synovial fluid without introducing additional fluorescence. Raman spectral analyses at 532 nm, 660 nm, 690 nm and 785 nm indicated that both asymptomatic and symptomatic samples had significant levels of fluorescence at excitation wavelengths below 700 nm, which either hindered the collection of Raman signal or necessitated prolonged durations of photobleaching. Raman-based diagnostics were more feasible at the longest excitation wavelength of 785 nm without employing photobleaching. This study further demonstrated that a near-infrared OEM based lower-cost Raman system at 785 nm excitation has sufficient sensitivity to identify crystals isolated from the synovial fluid. In conclusion, while lower excitation wavelengths

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provide greater signal, the fluorescence necessitates near-infrared wavelengths for Raman analysis of crystal species observed in synovial aspirates.

Keywords

Raman spectroscopy; fluorescence; absorbance; synovial fluid; monosodium urate monohydrate

Introduction

Monosodium urate monohydrate (MSU, leading to gout) and calcium pyrophosphate dihydrate (CPPD, leading to pseudogout) are the most frequently observed crystal types in the joint space. The inflammatory cells attempt to engulf and dissolve these crystals in a process called phagocytosis. However, cells rupture in a process called as the frustrated-phagocytosis, and the associated release of the intracellular digestive enzymes results in pain, tenderness, swelling and damage to bone, cartilage and the synovial lining. Gout affects 1–2% of the adult population and its incidence increases with age. [1,2] Accurate diagnosis of the crystal type is imperative to pursuing correct treatment. False negatives result in prolonged pain, continual damage to joints, time and resources lost for identifying other causes, and receiving medication for non-existing conditions. The impact of false positives on patient health can be worse than false negatives by promoting inappropriate long-term treatment and delaying the appropriate treatment.

Conclusive diagnosis of gout requires the analysis of the synovial fluid aspirates for the presence of crystals. MSU and CPPD crystals are birefringent and can be diagnosed by compensated polarized light microscopy (CPLM). However, only a small fraction of CPPD crystals are reported to be birefringent.[3] CPLM requires experienced operators who are available in major healthcare settings. However, the majority of gout is diagnosed in community based primary care settings based on symptoms only. Currently, false negative rate in gout diagnosis is in the range of 10%-30% (success rate depends on operators), while false positives occur up to 10%–20% range.[4–8] Therefore, an objective diagnosis system in community based primary care settings which would preclude a trained operator would be a significant improvement.

Raman spectrum serves as a chemical fingerprint in identifying components. It can also detect crystals which lack birefringence (such as calcium oxalates and basic calcium phosphates). Therefore, Raman spectroscopic analysis of synovial aspirates carries the potential for diagnosis of arthropathies [9,10]. McGill et al identified gout crystals in a synovial smear and a gouty tophus from a limited number of clinical samples using Raman analysis. [11] Maugars et al observed CPPD crystals in cartilage, muscle, and tendon sections using Raman microscopy. [12] Hawi et al have identified cholesterol crystals within cells resident in synovial aspirates. [13] These crystal-based studies utilized Raman spectroscopy in the microscopy mode which requires seeking for individual crystals visually on a large field of view. The strategy necessitates the utilization of research-grade Raman instruments with premium signal collection capability and, thus, limits the translation of the method to the clinical applications. Recently, sample preparation methods have been developed to congregate crystals at well-defined locations, enabling point-and-shoot Raman spectroscopy at clinically relevant crystal concentrations[14] where the diagnostic performance of Raman analysis compared favorably over CPLM in a limited number of clinical samples. Based on this premise, Raman spectroscopy based diagnosis of crystal species in synovial aspirates is beginning to gain feasibility.

A common challenge in Raman analysis of biological samples is the background fluorescence from the organic phase,^[15] which can limit or prohibit the relatively weaker Raman signal. While photobleaching mitigates organic based fluorescence to some extent, excessive fluorescence may require lengthy photobleaching durations that is clinically undesirable. Another venue to counter fluorescence is chemical bleaching where agents such as peroxide are used to bleach fluorophores. ^[16,17] While this treatment reduces the interference of fluorescence, it also affects the spectral profile and band ratios between Raman peaks recorded from peroxide treated tissues, as has been demonstrated for bone. ^[16] Furthermore, the peroxide treatment duration may be as long as several hours.^[16] This amount of time would be impractical in a clinical setting. Therefore, photobleaching or chemical bleaching are not ideal to tackle the fluorescence issue.

An alternative approach in dealing with background fluorescence is utilization of longer wavelength, e.g., near infrared (NIR) light to mitigate the generation. However, this generally comes together with a reduction in signal intensity because the Raman signal is inversely proportional to the fourth power of laser wavelength.^[18] Therefore, increasing excitation wavelength mitigates the background fluorescence at the expense of signal intensity. The purpose of this study was to analyze the background fluorescence and Raman signal in diagnosis of crystal species in synovial aspirates at different laser excitation wavelengths in the range of 532 nm – 785 nm with the goal of identifying a wavelength that is suitable for the analysis of synovial aspirates of symptomatic patients.

Material and methods

Sample collection and preparation

Synovial aspirates were collected from seven patients presenting “gout-like symptoms” and three asymptomatic synovial aspirates were obtained from Anatomy Gifts Registry (Hanover, MD) where the donors did not have any known joint disease history. Symptomatic sample collection was conducted under the approvals of Institutional Review Boards of institutions where synovial samples were collected (Metro Health Hospital, Cleveland, OH and Henry Ford Hospital, Detroit, MI). The patients presented to the clinic with gout-like symptoms and aspirates were collected as part of the normal diagnostic procedure. Clinical protocols for sample collection were described in an earlier report.^[14] Symptomatic samples had aggregates within the fluid whereas the asymptomatic samples were clear in appearance. The synovial fluid samples were frozen following collection and kept frozen during shipment and long-term storage at –20 °C.

Synovial fluid samples were digested with glycosaminoglycan and protein cleaving enzymes (hyaluronidase at 0.5 mg/ml and proteinase K at 1 mg/ml, Sigma-Aldrich) to release the crystals from the organic debris and also to reduce the viscosity of synovial fluid for ease of filtration, which was adapted from the previous work.^[14] The digested synovial fluid was transferred to a syringe and filtered through a custom-made filter cartridge mounted at the syringe tip. The filter cartridge was designed to guide and constrict the flow to a spot of polypropylene filter (30µm, EMD Millipore, Billerica, MA), retaining crystals over a 0.7 mm diameter spot for Raman analysis. The presence of MSU crystals obtained from clinical samples (needle-like, 2~20 µm in length)^[19] at the filtration site was confirmed by scanning electron microscopy (SEM) at a magnification of 2500x.

Synthetic MSU crystals were prepared following earlier protocols^[20,21] which yield crystals with similar size, morphology and birefringence to those found in gout^[14,22] as confirmed by compensated polarized imaging and X-ray diffraction earlier.^[14] Raman spectra were acquired from pure synthetic crystals to be used as reference for clinically obtained MSU crystals.

Fluorescence and absorption spectrometry

An absorption spectrometer/fluorometer (SpectraMax M2, Molecular Devices, CA) was used to record the absorption and fluorescence curves in 300 - 1000 nm and 500 - 850 nm (excited at 475 nm), respectively, at a resolution of 1 nm. A 1 J/flash Xenon flash lamp was used as the light source. A monochromator provides the excitation wavelength selection for fluorescence spectrum with bandwidth of 9 nm. Synovial fluid was held in a quartz cuvette (10 mm path-length, Starna Cells, CA) at a volume of 1 mL, and the corrections have been made for reflection and absorption from the quartz cuvette using deionized water for both absorption and fluorescence spectra. Spectra were recorded both before and after the digestion to investigate whether digestion procedure affects the fluorescence profile.

Microscopic autofluorescence imaging

A laser scanning confocal microscope (FV1000+IX81, Olympus America Inc, PA) was used to obtain fluorescence images of filtered samples. The samples were mounted on a microscope slide and excited with a 488 nm continuous wave laser with excitation power at the sample below 1 mW. A 10x objective with 0.4 numerical aperture (NA) (Olympus, Melville, NY) was used to focus the laser beam into the specimen. The autofluorescence signal was collected by the same objective and directed towards the internal spectral detector. Three broad spectral ranges were used for signal collection, 500 nm - 600 nm, 600 nm - 700 nm, and 700 nm - 800 nm. Each acquired image (512x512 pixels) covered an area of 1270x1270 μm^2 and was integrated over 3 frames to improve signal to noise ratio.

Raman spectroscopy

Raman analyses of samples were carried out by using custom-made Raman set-ups on an optical bench using interchangeable components to accommodate analysis at multiple wavelengths. The Raman shift measured by the systems was calibrated using the 520.7 cm^{-1} peak of a Si wafer. MSU crystals were identified by their characteristic peaks at 590 cm^{-1} and 631 cm^{-1} which respectively originate from the skeletal and breathing vibrations of the purine ring.^[23] Spectra of unfiltered, pure components were also acquired in powder form to confirm peak locations.

A custom Raman analysis system accommodated interchangeable laser wavelengths and associated filters to assess background fluorescence at various wavelengths (Fig. 1). A laser diode mount (LDM9T, Thorlabs, NJ) with integrated temperature control was used to drive multiple emission wavelength laser diodes (532, 660, 690 and 785 nm). A selection of dichroic mirrors, bandpass filters, and edge/notch filters from various vendors (Semrock, NY and Chroma, VT) were used in association with these excitation wavelengths. A 10x objective (0.25 NA, Olympus, Melville, NY) was used to focus the laser light resulting in a ~10 μm spot size. An f/4 spectrometer (HRS-VIS-25, Mightex, Toronto, Canada) with spectral dispersion of 2.0 $\text{cm}^{-1}/\text{pixel}$ and spectral resolution of 12 cm^{-1} was used to detect the Raman signal. If the baseline spectra saturated the CCD, measurement spot was photobleached and Raman spectra were collected at various intervals to affirm whether Raman signal can be obtained at a given wavelength following sufficient photobleaching.

In order to compare the Raman fluorescence levels from asymptomatic and symptomatic synovial fluids, three asymptomatic and three symptomatic filtration samples were investigated with a 785 nm OEM-Raman system (785L, Wasatch Photonics, NC). The OEM-Raman system included a 785 nm laser (Innovative Photonics Solutions, NJ) and an f/1.3 thermo-cooled spectrometer integrated with a NIR enhanced sensor (Hamamatsu S10420-1006, Bridgewater, NJ). This system was optimized and the spectral response was more than ten times stronger at 785 nm illumination than that of the previously described custom Raman system with interchangeable optics. A single lens with 25 mm focal length

was used to deliver the laser light on the sample over a $\sim 70\ \mu\text{m}$ spot. The CCD provided a spectral resolution of $10\ \text{cm}^{-1}$ and a spectral dispersion of $1.7\ \text{cm}^{-1}/\text{pixel}$ at $50\ \mu\text{m}$ slit width and using a gelatin based volume phase holographic transmission grating. Raman spectra were recorded from a symptomatic sample containing MSU crystals, pure synthetic MSU sample and a clean filter. Signal integration time and number of averages varied and such information is provided in the figure captions.

The background fluorescence at $650\ \text{cm}^{-1}$ was recorded at three randomly picked points in each filtration spot for each sample. The $650\ \text{cm}^{-1}$ was selected mainly because it constitutes the baseline of the major MSU peak located at $631\ \text{cm}^{-1}$. Mann-Whitney U Test was applied to test the differences between the asymptomatic and symptomatic samples and the level of significance was set as $P < 0.05$.

Results

The optical absorption spectra taken both before and after digestion of the three asymptomatic and the three symptomatic synovial fluids are shown in Fig. 2. A sharp absorption peak at $410\ \text{nm}$ was observed in two of symptomatic samples while this peak was mostly absent in the asymptomatic samples. Symptomatic synovial fluids presented higher absorption in visible light region than asymptomatic ones.

The corresponding fluorescence spectra displayed a major fluorescence peak at $515\ \text{nm}$ in one of the symptomatic samples and in all of the asymptomatic samples (Fig. 3a). The peak intensity was reduced after digestion. The fluorescence of one symptomatic synovial fluid deposited on the filter was taken by a laser scanning confocal microscope (Fig. 3b). It can be seen that the fluorescence was greatly reduced at $600\text{--}700\ \text{nm}$ range than at $500\text{--}600\ \text{nm}$ range, and further reduced by almost two orders of magnitude at $700\text{--}800\ \text{nm}$ range from its maximum value.

SEM imaging of MSU crystals filtered from synovial fluid using the filtration device confirmed the presence of needle-shaped crystals at the filtration deposit site (Fig. 4a). Raman spectra were collected at such deposits at 532 , 660 , 690 and $785\ \text{nm}$. The background fluorescence prevented the measurements to be performed at $532\ \text{nm}$, regardless of the duration of photobleaching. Raman spectra collected at $660\ \text{nm}$ excitation from filtered symptomatic samples displayed substantial amount of background fluorescence at the baseline which saturated the CCD. However, following $5\text{--}10$ minutes of photobleaching, it was possible to collect spectra at $660\ \text{nm}$. At $690\ \text{nm}$, Raman spectra from most samples could be acquired after 3 to 5 minutes of photobleaching after which MSU peaks could be observed (Fig. 4b, lower trace). Nevertheless, there were a few highly fluorescent samples from which the background fluorescence saturated the CCD even after 20 minutes of photobleaching (Fig. 4b, top trace).

Background fluorescence was present at $785\ \text{nm}$ excitation as well; however, it was not prohibitive as it was the case for the shorter wavelengths. For samples with the strongest fluorescence, the Raman spectrum with identifiable MSU peaks could be recorded immediately after the laser light was delivered to the sample using shorter integration times ($0.5\ \text{s}$) (Fig. 5a, inset). Spectral data demonstrated a reduction in fluorescence after it was continuously illuminated with the laser light. The intensity of background fluorescence decayed exponentially with the photobleaching time for $785\ \text{nm}$ excitation (Fig. 5a). An increase of spectral noise was observed with higher background fluorescence level (Fig. 5b). The signal intensity of the MSU peak did not change with the level of fluorescence. The background fluorescence intensities in Raman spectra collected at $785\ \text{nm}$ from

asymptomatic samples were significantly greater than symptomatic samples (asymptomatic: 20136 ± 9769 , symptomatic: 12468 ± 8460 , $P < 0.05$, Fig. 5c).

The Raman shifts at 590 and 631 cm^{-1} representing signature peaks of MSU crystals were evident in spectra of samples including crystals collected by the OEM Raman system over 1 second signal collection time with 10 averages and with no photobleaching. The spectral peaks of the filter material did not overlap with the peaks of interest of MSU (Fig. 6).

Discussion

The current study investigated the fluorescence from asymptomatic and symptomatic synovial aspirates in the context of Raman-based diagnosis of crystals leading to arthritic symptoms in the joint space. Following digestion and filtration steps, crystals were extracted from synovial aspirates and collected over submillimeter spots for point-and-shoot Raman spectroscopy. Digestion was essential for several purposes. First, the organic debris/aggregates included crystals which were released to the fluid after digestion, increasing the crystal recovery efficiency. Second, the hyaluronic acid phase imparts viscosity to the synovial fluid which in turn makes syringe filtration hard. Digestion of the hyaluronic acid was observed to decrease the viscosity and allow filtration. Fluorescence and absorbance were conducted both before and after digestion to assess whether the addition of enzymes affected the absorbance/fluorescence profiles.

Synovial fluid is a complex mixture that contains water, hyaluronic acid, ions, proteins, and cells. Physiologically normal synovial fluid is transparent and clean^[24]. The composition of synovial fluid from symptomatic patients is much more complex due to inflammatory processes and overabundance of cells. The appearance of symptomatic synovial fluid is frequently turbid with presence of organic aggregates and, occasionally blood.^[24,25] The absorption peak at 410 nm (Fig. 2) is attributed to haemoglobin, according to Stone et al.^[26] This absorption peak was more strongly present in symptomatic samples than asymptomatic ones. The greater presence of blood in symptomatic samples can be due to greater level of inflammation and wear in diseased joints. It must be emphasized that the symptomatic samples were obtained from patients whereas asymptomatic samples originated from post-mortem cadavers which may have resulted in greater level of blood in symptomatic samples.

For all samples, the absorbance decreased as the wavelength increased, and after 700 nm , the absorbance was $\ll 1$ for all samples. This absorbance reduction is partially due to the decreased scattering associated with the scale distribution of microscopic particles such as proteins, lipids and cells.^[26] Assuming large contribution from scattering to absorbance measurements, the significantly higher absorbance for symptomatic samples than asymptomatic ones indicated a higher density of particles in the synovial fluid from diseased joints. Therefore, highly concentrated MSU crystals observed in symptomatic #1 sample could explain its higher absorbance baseline than other symptomatic samples mainly due to the scattering from MSU crystals. This conclusion is based on the baseline which became comparable with crystal-free samples after the removal of crystals (data not shown). Overall, these observations imply that absorbance and fluorescence profiles may be able to discriminate asymptomatic and symptomatic synovial fluids. However, current observations need to be substantiated on a larger sample set.

In biological samples, the fluorescence is often associated with the aromatic groups in folded proteins, like tryptophan, tyrosine, and phenylalanine, and locations of these groups in proteins result in variation of fluorescence.^[27] The fluorescence intensity varied between individuals, regardless of whether the samples were asymptomatic or symptomatic in origin. A fluorescence peak at 515 nm was observed in all asymptomatic samples and in one

symptomatic sample. While the source of this peak is unknown at this time, it can be speculated to originate from the hyaluronic acid, which is reported to be more heavily present in asymptomatic samples.^[28] The fluorescence spectrum was recorded for a sample before and after removal of crystals via filtration (data not shown) so as to determine whether MSU crystals contribute to the fluorescence. It was observed that the fluorescence did not change following the removal of crystals; therefore, as expected, crystals do not contribute to the observed fluorescence.

Enzymatic digestion of samples resulted in slightly increased absorbance in asymptomatic samples whereas absorbance in symptomatic samples decreased notably following digestion (Fig. 2). Particulate aggregates in the symptomatic synovial fluid were cleaved into smaller pieces by digestion which may have attenuated the scattering effects and led to an overall decrease of absorbance profile for symptomatic synovial fluid. The observed increase of absorbance in asymptomatic fluids following the digestion is unclear. Since asymptomatic fluid lacks organic debris and is dominated by hyaluronic acid, it appears that the digestion of hyaluronic acid changes the conformation and size of this glycosaminoglycan in a fashion to increase scattering, and thus, the measured absorbance. Regardless of the dichotomy on the effects of digestion on absorbance of symptomatic versus asymptomatic samples, fluorescence in 500 nm to 600 nm range was reduced in all asymptomatic and one symptomatic samples following digestion (Fig. 3a).

Although digestion and filtration dissolves and reduces the organic debris burden, there is an unpredicted amount of organic material left on the filtrated spot including MSU crystals. The organic debris burden is greater in symptomatic fluid than in asymptomatic fluid to the extent that the former had visible particulate debris and the latter was clear. Based on this difference, one would expect a greater level of fluorescence from symptomatic fluid than from asymptomatic fluid. In contrast to this expectation, fluids from symptomatic patients tended to have lower fluorescence as observed by fluorescence spectra and Raman spectra. Based on this observation, it appears that the fluorescence is not solely driven as intensely by the organic debris observed in fluid collected from diseased joint. Rather, it appears that the hyaluronic acid that is more prominent in asymptomatic fluid^[28] is a contributor to fluorescence and that hyaluronic acid may exist in lower amounts and/or in a different conformation in symptomatic samples to an extent that results in lower fluorescence. However, these assertions need to be proven by further experiments measuring the relative amounts of protein and hyaluronic acid in these sample populations.

The results shown in Fig. 3a and 3b suggested that fluorescence interference on Raman spectroscopy of synovial fluid may be overcome by using excitation wavelength longer than 660 nm. For this reason, we investigated Raman system for synovial fluids based on lasers at 660 nm, 690 nm, and 785 nm, aiming to find a compromise between high spectral response at shorter wavelength and less fluorescence at longer wavelength. Results showed that, at 690 nm excitation, for most clinical samples prepared following our protocol, the fluorescence has been reduced to a level that enabled the collection of Raman spectral data with less than 5 minutes of photobleaching (Fig. 4b). However, several samples still displayed extremely strong fluorescence that saturated the spectrometer that did not bleach at durations too lengthy for practical clinical application. Therefore, utilization of lasers at greater wavelengths than 690 nm is advisable.

Raman spectrum of MSU crystals isolated from a clinical sample can be observed successfully at 785 nm for both the custom Raman system and the OEM Raman system. The main difference between the two systems was that the OEM Raman system contained a NIR enhanced spectrometer with a significantly higher quantum efficiency than that of the custom system (>55% vs. < 20% quantum efficiency at 830 nm). In addition, the CCD

sensor of the OEM system was thermoelectrically cooled to 15 °C, which reduced the system noise; the volume phase holographic transmission grating also provides as high as 90% diffraction efficiency. The OEM system had twenty-fold better performance than the custom system in terms of signal to noise (S/N) ratio (Fig. 5d). This would be expected to improve the limit of detection in future clinical applications.

Despite the fact that 785 nm wavelength performed most favorably in the wavelength range we studied, fluorescence was still present. At short signal integration durations (less than 0.5 second), signal could be collected directly without any photobleaching. Therefore, one option for data acquisition is to employ short integration times averaged many times without photobleaching. However, time dependent fluorescence (Fig. 5a) and fluorescence dependent noise studies (Fig. 5b) showed that signal to noise ratio improved with reduced fluorescence. Therefore, even at 785 nm, brief photobleaching periods (within 1 minute) are advisable to improve the S/N ratio.

While we demonstrated that 785 nm is suitable, the current study did not analyze the range of 700 nm – 785 nm mainly due to lack of cost-efficient laser-diodes that provide reasonable specifications (stability, bandwidth etc.) for Raman analysis. It may be possible that a wavelength in this range may address the fluorescence while providing more signal than 785 nm. However, since 785 nm has become a standard wavelength in Raman analysis, optical components are more readily available, making 785 nm more attractive as a candidate towards integration of a diagnostic device.

Conclusion

The current study investigated the fluorescence from asymptomatic and symptomatic synovial aspirates in the context of Raman-based diagnosis of crystals leading to arthritic symptoms in the joint space. It was demonstrated that filtering of the samples following enzymatic digestion allowed the utilization of an inexpensive Raman set-up using OEM-based off-the shelf components. In addition, we showed that the Raman signal can be recovered from crystals at 785 nm laser excitation without being masked by background fluorescence from the organic phase. Given that Raman-based diagnosis is definitive and that it is objective, the method developed here can be applied to a large clinical sample set to assess Raman spectroscopy's promise as a point of care convenient diagnostic tool.

Acknowledgments

The authors thank Emma Barnboym and Dianne Morus (at MetroHealth Medical Center) and Daniel Oravec (at Henry Ford Health System) for coordination of the collection of clinical samples. We also thank the Divisions of Rheumatology at both clinical centers for identifying and recruiting patients. This study was funded by the research grant R01AR057812 (OA) from the NIAMS institute of NIH. This publication was also made possible by the Clinical and Translational Science Collaborative of Cleveland, UL1TR000439 and its Clinical Research Unit core based at MetroHealth Medical Center. The UL1TR000439 is awarded by the National Center for Advancing Translational Sciences (NCATS) component of the National Institutes of Health and NIH roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Figure 1. Schematic diagram of the optical layout and basic components of the low-fidelity custom Raman set-up. The components included F: laser line filter, DM: dichroic mirror, OBJ: objective lens, M: mirror, NF: notch filter, L: lens, sample holder and spectrometer.

Figure 2.

The absorbance spectra of synovial fluids. Data were taken from as-retrieved synovial fluids collected from asymptomatic and symptomatic patients, also, before and after digestion.

Figure 3.

(a) Corresponding fluorescence spectra of the samples in Fig. 2, with excitation wavelength set at 475 nm. (b) Images of a symptomatic synovial fluid deposited on a filter from a laser scanning confocal microscope, with the emission window set at 500–600, 600–700 nm, and 700–800nm, respectively.

Figure 4.

(a) SEM image of MSU crystals isolated from a clinical sample deposited on filter (x2,500 magnification). (b) Representative Raman spectra from a highly fluorescent symptomatic MSU sample recorded by the custom Raman setup at 690 nm (25 mW, signal integration time 6 seconds, no averages), after 3 minutes and 20 minutes of photobleaching, respectively. Red arrows indicate the signature MSU peaks.

Figure 5.

Dependence of fluorescence on time (a) and noise level dependence on fluorescence (b) of high fluorescent synovial fluid deposited on a filter measured by the OEM Raman system at 785 nm (integration time 0.5 s , laser power 60 mW). The noise level is determined by the standard deviation of the peak-free region at $680\text{--}740 \text{ cm}^{-1}$ following the baseline correction. The inset in (a) shows the spectra corresponding to the first and the last data points in the main plot. (c) Background fluorescence intensities at 650 cm^{-1} for asymptomatic and symptomatic samples. (d) Typical Raman spectra acquired by the custom and the OEM systems at 785 nm excitation from a typical clinical sample with MSU

concentration under the same data acquisition conditions. The laser power was 40 mW, with integration time of 6 second averaged 16 times.

Figure 6. Representative Raman spectra of clinical MSU crystals deposited on polypropylene filter, as well as pure MSU crystals and a clean filter. Spectra were recorded by the OEM Raman setup with 785 nm excitation. The spectra were vertically shifted following background fluorescence correction for better viewing. The laser power was 60 mW and the integration time was 1 second and averaged 10 times.