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REVIEW ARTICLE

# Raman spectroscopy applications in rheumatology

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## Abstract

Raman spectroscopy is a type of vibrational spectroscopy based on the inelastic scattering of photons, which has attracted much attention due to its potential clinical application in rheumatology. In this review, we discuss the typical spectral features of cartilage, bone, synovial fluid, and pathologic crystal deposits, as well as methods of amplifying the Raman signal of biofluids such as drop-coating deposition Raman spectroscopy. Further, applications of Raman and drop-coating deposition Raman spectroscopy in osteoarthritis are described, highlighting the clinical potential of these methods. We also discuss the role of Raman and related techniques in analyzing pathologic crystals such as monosodium urate, calcium pyrophosphate dihydrate, and hydroxyapatite. The results presented in this review demonstrate that Raman spectroscopy has grown past the stage of proof-of-concept, especially in the case of pathologies involving crystal depositions such as gout and calcium pyrophosphate deposition disease , for which the method has been validated on large number of samples. As the medical community becomes more and more aware of Raman spectroscopy, it is envisioned that it will become a standard technique in the near future.

**Keywords** Rheumatology · Raman spectroscopy · Osteoarthritis · Gout · Calcium pyrophosphate deposition disease · Ectopic calcifications

## Introduction

Chronic pathologies of the musculoskeletal system that affect the joint and the surrounding tissues represent a major cause of disability worldwide. More developed countries, characterized by an aging population, are particularly affected. Common musculoskeletal diseases include osteoarthritis (OA), rheumatoid arthritis, gout, and calcium pyrophosphate dihydrate (CPPD) deposition disease (also known as chondrocalcinosis or pseudogout) [1]. The pathogenesis, diagnosis, and treatment of these conditions represent a vibrant topic of research, aimed at increasing the quality of life while decreasing the healthcare costs [2].

Biophotonics is an emerging field that exploits optical techniques, including Raman spectroscopy, for advancing knowledge in life sciences [3]. Raman spectroscopy relies on the interaction of laser photons with molecules for inquiring the chemical composition [3]. Molecular structural information is gained by shining the laser on the sample and then collecting the scattered laser photons onto sensible detectors. The detectors measure the shift in wave number determined by the interaction of the laser photons with the vibrational modes of molecules within the sample. For most biological applications, the lasers are in the visible or near-infrared range.

Raman spectroscopy should be regarded as a complementary method to Fourier-Transform Infrared Spectroscopy (FTIR) [3], since often Raman active bands are weak FTIR bands, and vice-versa. Although both Raman and FTIR yield information about the vibrational modes in the system, FTIR spectroscopy is based on the energy resonance of the photons with the energy difference between vibrational modes of molecules, whereas, Raman spectroscopy relies on the inelastic scattering of photons by molecules.

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FTIR has been widely used for assessing bone and mineralized tissues [4], but compared to FTIR, Raman spectroscopy features some unique properties which have attracted much attention due to their potential clinical application. First, Raman spectroscopy is not hindered by the spectra of water molecules, which enable in vivo measurements. Moreover, the signal can be acquired through optical fibers, making Raman spectroscopy amenable for endoscopic evaluations (including arthroscopy). Second, Raman spectroscopy can be coupled to a regular microscope in a technique termed Raman microspectroscopy, which gives detailed spatial information about the molecular content of the samples. Third, the intensity of the Raman signal can be amplified up to several orders of magnitude by adsorbing the molecules onto metal nanostructures in a method called surface-enhanced Raman scattering (SERS) [5]. Considering that Raman and related techniques have shown promising results in several pilot studies involving almost every component of the joint (see below), it is expected that the method will become soon a widely used technique for the evaluation of the joint pathology.

The joint is a complex structure, regarded as a self-contained organ that includes bone, articular cartilage, meniscus, ligaments, and synovial fluid (SF) [6]. The proper functioning of the joint requires a complex chemical composition and supramolecular architecture, the details of which are just beginning to be unraveled. Raman spectroscopy has been increasingly used to explore both the functioning and the pathological modifications of these tissues. The purpose of this manuscript is to give a comprehensive dictionary regarding the biochemical information that can be gained from the Raman signal of bone, cartilage, SF, and pathologic crystal deposits such as CPPD and monosodium urate (MSU).

## Raman signal of bone and pathologic calcifications

The Raman signal of bone reflects its two main constituting elements: a mineral phase, which is represented by a special form of apatite and an organic matrix, which is dominated by type I collagen [6]. The collagen fibbers consist of a triple helix formed by intertwining polypeptide strands called alpha chains, which are rich in glycine, proline, and hydroxyproline [7]. The collagen strands have crosslinked non-helical terminal domains, the presence which can be evaluated using Raman spectroscopy. The bone apatite differs from the pure hydroxyapatite found throughout nature by carbonate ($CO_3^{2-}$) and hydrogen phosphate ($HPO_4^{2-}$) substitutions [8]. The mineral phase is spectrally apparent as the 959 $cm^{-1}$ band of phosphate ($\upsilon_1$ $PO_4^{3-}$). The peak is shifted towards lower wavelengths in the newly deposited bone, due to higher hydrogen phosphate content. The carbonate moiety of the crystal lattice can be assessed based on a characteristic Raman band around 1070 $cm^{-1}$ ($\upsilon_1$ $CO_3^{2-}$). Pathologic calcifications such as those found in CPPD deposition disease also yield a Raman spectrum similar to that of hydroxyapatite substituted by carbonate and hydrogen phosphate.

The Raman bands of the bone organic matrix, attributed almost exclusively to type I collagen, are represented by the amide I (1660–1680 $cm^{-1}$), amide III (1240–1270 $cm^{-1}$), and $CH_2$ deformations (1450 $cm^{-1}$), and two bands stemming from proline (850 and 920 $cm^{-1}$) and hydroxyproline (875 $cm^{-1}$). The absolute intensity of the bands is almost never reported, since it can vary widely between measurements. Therefore, it is customary to express the spectral information in terms of band intensity/area rations. These ratios, which combine two or more bands, can give comprehensive information about the chemical properties of the bone and they can regard: (i) the mineral to organic ratio, (ii) the mineral component, or (iii) the organic component.

This most widely reported parameter is the mineral to matrix ration (MTMR), which should be conceived as a spectral equivalent of the bone matrix density (BMD) used in the clinical setting [9]. The mineral phase is almost always represented by the 959 $cm^{-1}$ $\upsilon_1$ $PO_4^{3-}$, but for the organic matrix the candidates include almost any band attributed to collagen such as the amide I (1660–1680 $cm^{-1}$), amide III (1240–1270 $cm^{-1}$), and $CH_2$ deformations (1450 $cm^{-1}$). Other parameters that regard the organic components are represented by the collagen crosslinks, which can be deduced either from the amide I ratio (1660 $cm^{-1}$/1680 $cm^{-1}$) or amide III ratio (1240 $cm^{-1}$/1270 $cm^{-1}$), but there are only few studies which validated these metrics. The mineral stoichiometry is usually described by the carbonate to phosphate ratio (1070 $cm^{-1}$/959 $cm^{-1}$). A comprehensive discussion regarding the parameters described here and other less frequently employed ratios can be found in the excellent review by Esmonde-White [6].

## Articular cartilage

Articular cartilage is a viscoelastic connective tissue, which delineates the articular space of synovial joints [10]. Articular cartilage provides resistance to compressive forces, and together with the SF, it enables the frictionless movement of the joint. As opposed to the bone, cartilage is a non-mineralized tissue which contains predominantly type II collagen instead of type I collagen [7]. Cartilage is also very rich in large water-binding molecules called glycosaminoglycans (GAGs), which are responsible for the elastic properties of the cartilage. It is very interesting to note that the Raman spectra of articular surface shares several bands with those of the bone, which are observed even in points in which the articular surface is intact. Therefore, aside from a band at 1063 $cm^{-1}$ due to GAGs (chondroitin sulphate), the Raman spectrum of cartilage is similar to that of bone in the in vivo setting [6]. This phenomenon is explained by the fact that the cartilage is a thin, strongly scattering layer, which cannot prevent the lasers from reaching the underlying bone. To exploit this phenomenon, several authors used Raman

spectroscopy to gain information about the underlying subchondral bone (see below), which is known to play important roles in several conditions.

### Synovial fluid

SF is a plasma filtrate (dialysate) enriched in hyaluronic acid, which functions as a lubricant and shock absorbent within the joint and which aids the nutrition of the cartilage by transporting glucose and other nutrients [11]. Considering that SF aspiration is done on a regular basis for diagnostic purposes [12], there is a great opportunity for Raman spectroscopy and related techniques in providing additional information regarding the presence of pathological conditions. To amplify the Raman signal, the SF can be dried onto a solid substrate in a method termed drop coating deposition Raman spectroscopy, which exploits the "coffee ring" effect [13]. While drying, the molecules present in the SF are concentrated and suffer a coarse separation based on their solubility: the more soluble molecules will be deposited in the center while less soluble molecules (mainly proteins) will be deposited first at the periphery of the spot. Therefore, the Raman spectra acquired form the periphery of the SF spot is often dominated by bands assigned to proteins such as the phenylalanine Raman band around 1000 $cm^{-1}$, amide I (1660–1680 $cm^{-1}$), or amide III (1240–1270 $cm^{-1}$).

Besides detecting perturbations in the tissues that comprise the joint, Raman spectroscopy is also a potential tool for analyzing pathologic crystal deposits such as CPPD or monosodium urate (MSU). MSU and CPPD can be differentiated based on compensated polarized light microscopy (CPLM), a technique that is not always available. The differentiation between the two can be achieved using their characteristic Raman bands, of which the most intense are at 631 $cm^{-1}$ for MSU and 1050 $cm^{-1}$ for CPPD [14].

## Clinical applications of Raman spectroscopy

### Osteoarthritis

Aging is the main risk factor for primary OA, but joint senescence and OA are distinct processes. For example, synovial inflammation, subchondral bone thickening, and the development of chondrocyte clusters represent OA-specific processes, which are not seen during normal aging [15]. However, OA seems to hijack and amplify several molecular pathways involved in the aging process, such as cellular senescence, dysregulated nutrient sensing, and mitochondrial dysfunction, which leads to the damage of the joint and to functional impotence and pain. Detailed guidelines on the diagnosis and treatment of knee or hip OA have been issued by professional societies, including the European League Against Rheumatism (EULAR) [16, 17]. Considering that OA is frequently diagnosed only after the onset of irreversible joint damage, several studies have focused on establishing tools to identify early signs of OA.

For instance, Esmonde-White et al. developed a custom-made pen-like Raman probe in a proof of principle study that sought to adapt Raman spectroscopy for arthroscopy [18]. First, the authors obtained high-quality reference Raman spectra of intact articular surface and of exposed subchondral and cancellous bone from the proximal radius of a donated arm. The proximal radius was chosen since it is less frequently affected by OA lesions. Next, the authors designed a Raman probe, which consisted of several excitation and collection fibbers distributed over two concentric rings. The probe was tested on two-knee articular surfaces retrieved from a single donor which presented intact cartilage, focal lesions, and full-thickness erosions. The authors focused on the 900–1150 $cm^{-1}$ spectral region, where they described several spectral modifications that accompanied the transition from intact cartilage to focal lesions to erosions: the disappearance of the 1065 $cm^{-1}$ Raman band of chondroitin sulphate, which was replaced by the $\upsilon_1$ $CO_3^{2-}$ 1070 $cm^{-1}$ Raman band of the B-type carbonate and an increase in the intensity of the phosphate $\upsilon_1$ $PO_4^{3-}$ 958 $cm^{-1}$ band. The authors also found that lesions were associated with a decrease in the cartilage to bone ratio (1063 $cm^{-1}$/958 $cm^{-1}$) and an increase in the bone to collagen ratio (958 $cm^{-1}$/920 $cm^{-1}$). In order to understand the effect of cartilage thickness on the collected Raman signal, the authors employed tissue phantoms and a computer simulation method called finite element analysis. These models suggested that cartilage thickness has a complex effect on the sampling efficiency of bone and cartilage, which can perturb the interpretation of the Raman spectra. The authors suggest that this confounding factor could be eliminated by assessing the actual thickness of the cartilage by means of magnetic resonance imaging (MRI), ultrasound, or optical coherence tomography (OCT), or by employing multiple source-detector offsets.

The spectral pattern that accompanies the progression of cartilage damage was also assessed by Takahashi et al., who used a confocal Raman microspectrometer to study the spectral differences between one healthy and five damaged knee articular surfaces [19]. The authors were interested in the correlation between macroscopic visual assessments of the degree of damage (the Collins scale) and the Raman spectra. The results showed that the degree of damage was correlated with an increase in amide III doublet ratio (1241 $cm^{-1}$/1269 $cm^{-1}$), which suggests that OA leads to an increase in collagen randomness. The most important differences were observed between grade I and grade II OA, suggesting that the progression of the disease implies important chemical modifications between these stages. Similar results were reported by Kumar et al. on formalin-fixed knee articular surface samples from three human knees, which were assessed vissualy in terms of the damage according to the International Cartilage Repair Society (ICRS) scale (I to IV) [20]. The

authors were interested in the cartilage damage, therefore they excluded grade IV lesions (full thickness lesions) from their analysis. The results confirmed the previous study by Takahashi et al. regarding the initial alteration in the amide III ratio and showed that the intensity ratio 1245 $cm^{-1}$/1270 $cm^{-1}$ increases with the ICRS grade and that there was statistically significant difference ($p < 0.05$) between grades I and II and between grades I and III but not between grades II and III. Moreover, the authors also found that the cartilage specific 1064 $cm^{-1}$ Raman band of the proteoglycan is changed in the more advanced stages of the disease (significant differences between grade I and III, grade II and III, but not between grade I and II). The authors also employed multivariate statistical methods, which could predict the IRCS grade with an overall efficiency of 85%. These results must be interpreted with caution, since these results obtained on formalin-fixed tissues cannot be directly extrapolated to arthroscopic findings. Moreover, the authors performed the Raman study on pieces of excised cartilage surface that included the subchondral bone but not underlying bone, an experimental setup which differs from in vivo conditions.

Apart from the cartilage surface, several studies were specifically interested in the chemical changes of the underlying subchondral and cancellous bone of OA patients. The subchondral bone is certainly affected during OA progression, a phenomenon evidenced by the appearance of radiologic lesions. However, it is not clear weather subchondral bone damage is secondary to cartilage disruption, or it represents a distinct event which develops simultaneously or even before the actual joint surface alteration. To clarify this issue, several studies took hip and knee joints retrieved from OA patients and cut them to expose the subchondral bone. For instance, the results of a study by Buchwald et al. [21], which was done on the femoral heads retrieved from 10 OA patients and 10 controls, showed a significant overall decrease in the MTMR (via amide III) and collagen randomness (via amide III) and an increase in the mineral stoichiometry for the subchondral bone of OA femoral heads. The unexpected finding was that there was no difference between most weight-bearing regions of the joint (i.e. most damaged) and the least weight bearing regions (i.e. least damaged) in terms of MTMR and collagen randomness, a finding which suggests an intrinsic subchondral bone damage. The study has also showed that there are no spectral differences in the spongy bone between OA and control samples. A similar study on tibial plateaus was performed by Kerns et al. [22], which compared the most weight and least weight bearing parts of proximal tibial plateaus retrieved from 10 OA patients and 10 controls. Interestingly, in contradiction with the study by Kerns et al., the results showed an overall increase in the MTMR (via amide I) based on combined spectral data of medial and lateral compartments of the joint. Nonetheless, the study confirmed the lack of spectral differences between the medial (most weight-bearing part) and the lateral site (least weight-bearing site).

A more recent study by Tomanik et al. [23] on 9 tibial plateaus retrieved from OA patients showed that the progression of the disease is accompanied by a decrease in the MTMR. However, this was an uncontrolled study and all results were compared to the early stage OA. In terms of animal models, the results regarding the MTMR are also contradicting, since one study by Dehring et al. [24] and one by Yu et al. [25] showed a reduced MTMR (via $CH_2$ deformation and proline, respectively), de Souza et al. [26] showed an increase in the MTMR (via amide III), while Mangueira et al. [27] could not detect any differences. However, it is hard to compare these studies because of differences in the animal model of OA (rats or rabbits), in the mode of inducing the damage (collagen transgenic mouse, treadmill strenuous exercise, collagenase, or medial meniscal tear) as well as in the way of defining the mineral to matrix ratio. These results demonstrate that in order to gain comprehensive results from the joint cartilage with Raman spectroscopy, there should be a more uniform protocol in reporting the data, since the presence of cartilage tissue seems to greatly complicate the Raman signal acquired from the underlying bone. In the cases where the Raman signal was acquired directly from the cancellous bone, the results are easier to interpret. For example, in a series of three studies published by a group from Rochester University on a transgenic murine model of glucocorticoid treated rheumatoid arthritis [28–30], the authors showed that the Raman spectra acquired from the bone clearly outperforms BMD in terms of fracture risk prediction. These promising results lay the foundation for translating Raman spectroscopy in the clinical practice.

The SF has also been studied using drop coating deposition Raman spectroscopy, in an attempt to predict OA damage based on the spectral modifications. In a series of papers published by a group from Michigan University [31, 32], which culminated with a study on 40 patients with varying degrees of joint damage, the authors showed that the more damaged patients have a statistically significant increase in the 1080 $cm^{-1}$/1001 $cm^{-1}$ ratio and amide I band intensity ratio (1670 $cm^{-1}$/1655 $cm^{-1}$). Using unsupervised methods, the authors could classify samples from damage (high-grade OA) and no damage (low-grade OA) patients with a sensitivity of 74% and a specificity of 71%.

The cellular component of the cartilage, namely the chondrocytes, was also assessed for capturing the spectral modifications that accompany OA progression [33]. The human chondrocyte cells retrieved from OA patients were cultured and then probed using Raman microspectroscopy. The authors successfully predicted the degree of damage (ICRS grades of osteoarthritic cartilage) based on Raman features with an overall accuracy of 92.2%. The spectra indicated that chondrocytes derived from severely affected patients (high

ICRS grade) where characterized by a general decrease in protein content, as inferred from the amide I, amide III, and phenylalanine bands. The decrease in protein content was also accompanied by a decrease in the intensity of the bands assigned to nucleic acids (785 $cm^{-1}$), probably due to an increase in the internucleosomal DNA cleavage during the progression of disease.

## Gout, CPPD disease and ectopic calcifications

Gout is the most prevalent form of inflammatory arthritis, which affects about 2–4% of adults [34]. An important differential diagnosis of gout is represented by CPPD deposition disease, which sometimes can present with similar symptoms (acute arthritis) [35]. The diagnosis of gout and CPPD deposition disease is confirmed by the presence of MSU and CPPD crystals in the SF by means of CPLM [36, 37]. However, the technique is not always available. Therefore, other methods of detecting MSU and CPPD are needed. The possibility to differentiate between MSU and CPPD lesions based on Raman was first reported by McGill et al., which analyzed MSU deposits from a gouty tophus and synovial tissue from a patient diagnosed with chondrocalcinosis that suffered total knee replacement [38]. Thus, the authors demonstrated that based on the two characteristic bands at 631 $cm^{-1}$ for MSU and 1050 $cm^{-1}$ for CPPD crystals, Raman spectroscopy can differentiate between the two types of crystals.

More recently, a group from Case Western Reserve University (Cleveland), developed a shoe-box sized Raman spectrometer which was able to detect MSU and CPPD crystals from SF [14, 39, 40]. After a digestion step, the crystals were concentrated by microfiltration in a well-defined spot which enabled point-and-shoot Raman spectra acquisition. The device was validated against CPLM on 174 SF samples from symptomatic patients. The CPLM found 56 samples positive for crystals (44 MSU and 12 CPPD). The overall agreement of the device with CPLM was approximately 90%. The authors refrained from deriving sensitivity or specificity values for their device, since CPLM is known to be unreliable. The authors stressed that their device is not intended to replace CPLM, but only to aid the diagnosis when there is a lack of skilled technicians.

A recent study by Abhishek et al. [41] showed that Raman spectra acquired directly from above the medial aspect of the first metatarsophalangeal joint showed MSU signal in 7 out of 10 patients diagnosed with gout and in 1 out of 10 controls. Since the authors did not check the presence of MSU crystals with other established methods and the patients were on urate-lowering drugs, the authors' opinion was that the joints of the three patients classified incorectly were MSU-free. In addition, the authors suggested that one control subject displayed asymptomatic MSU crystal deposits. These examples show that Raman spectroscopy has grown into a mature field and that it is expected to enter the clinics soon.

Interestingly, several studies have shown a link between OA and the presence of CPPD or other pathologic crystals in joint cartilage and SF. In a study by Nalbant et al. [42], out of the 330 patients diagnosed with OA, 52% had crystals in their SF (21% CPPD crystals, 47% had basic calcium phosphate crystals, and 16% had both types of crystals). Moreover, the presence of crystals was correlated with the severity of OA. Therefore, Fuerst et al. developed an experimental algorithm for identifying and quantifying the presence of pathologic calcifications in excised joints using a sequence of digital contact radiography (DCR), scanning electron microscopy (SEM), X-ray element analysis, and Raman spectroscopy. Thus, the authors successfully identified CPPD (using its characteristic peak at 1050 $cm^{-1}$) in the tibial plateau of one patient and hydroxyapatite deposits in other three [43]. The authors advocate for more epidemiologic studies regarding the implications of pathologic calcifications in OA. Meniscal calcifications were evaluated in a rabbit model of early osteoarthritis by Levillain et al. [44]. The hypothesis of the study was that menisci could supply important information regarding the onset of OA. Even though the authors did show modifications in the micromechanical properties and in the GAG content of menisci, Raman spectroscopy failed to show spectral features associated with early OA.

Raman spectroscopy has also been employed for analyzing calcific tendinitis [45]. The diagnosis of calcific tendinitis is made based on imaging findings, and the treatment can range from conservative to surgical excision. Historically, the deposits were considered to be related to previous trauma or ischemic lesions of the tendon. However, Urist et al. and Uhthoff et al. suggested that the tendon suffers a fibrocartilaginous metaplasia, which leads to the deposition of calcific lesions [46, 47]. The authors also proposed based on histology findings that the disease represents a cyclic, step-wise process which includes a precalcific, calcific, and postcalcific stage. Raman and FTIR-spectroscopy data on calcific tendinitis was reported by Chiou et al., which showed that the stage of the lesion correlates with the chemical composition of the crystal deposits assessed via Raman and FTIR spectroscopy [48]. Thus, based on the deconvolution of the $\upsilon_1$ $CO_3^{2-}$ band, the authors showed that the progressive calcification of the lesion is accompanied by a shift in the $\upsilon_1$ $CO_3^{2-}$ band from 866 $cm^{-1}$ to 871 $cm^{-1}$, since precalcific lesions consist of poorly organized crystal structures with liable carbonates exhibiting the $\upsilon_1$ $CO_3^{2-}$ band at 866 $cm^{-1}$, while the highly organized crystals found in late stage lesions consist of B-type carbonated apatite exhibiting the $\upsilon_1$ $CO_3^{2-}$ band at 871 $cm^{-1}$. Moreover, the chemical composition also correlated with the shape of the lesions on ultrasound as well as with the clinical symptoms (pain).

## Conclusion and perspective

Raman spectroscopy is an emerging technique with enormous potential for aiding the diagnosis, grading and follow-up of diseases such as osteoarthritis, gout, pseudogout, or calcific tendinitis. However, the translation of these techniques in the point-of-care setting will require substantial effort for homogenizing the experimental setup and for increasing the reproducibility of the experiments. This issue will certainly be mitigated in the future through the advances in the technology of fabricating optic devices and detectors. Moreover, the advent of microfluidics and lab-on-a-chip devices as well as the miniaturization of Raman spectroscopes and a significant improvement in their cost effectiveness will further facilitate the translations of Raman spectroscopy and related techniques in the clinical setting.

Another important obstacle in the clinical implementation of Raman spectroscopy is represented by the gap between physicians and spectroscopists, with the former not being aware of the possibilities put forward by Raman spectroscopy and the later not being able to conduct clinically rigorous validation. As the gap between the two sides closes through international collaborations such as the European COST Action Raman4Clinics or The International Society for Clinical Spectroscopy (CLIRSPEC), Raman spectroscopy is envisioned as a routine technique of future healthcare.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.



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