analytical chemistry

Letter

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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.0c00290 • Publication Date (Web): 14 May 2020

Nanometric chemical speciation of abnormal deposits in kidney biopsy: Infrared-nanospectroscopy reveals heterogeneities within vancomycin casts.

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Abstract (148 words)
InfraRed (IR) spectromicroscopy allows chemical mapping of kidney biopsy. It is particularly interesting for chemical speciation of abnormal tubular deposits and calcification. In 2017 using IR spectromicroscopy, we described a new entity called vancomycin cast nephropathy. However, despite recent progresses IR microspectrometer spatial resolution is intrinsically limited by diffraction (few micrometers). Combining atomic force microscopy and IR lasers (AFMIR) allows acquisition of infrared absorption spectra with a resolution and sensitivity in between 10 and 100 nm. Here we show that AFMIR can be used on standard paraffin embedded kidney biopsies. Vancomycin cast could be identified in a damaged tubule. Interestingly unlike standard IR spectromicroscopy, AFMIR revealed heterogeneity of the deposits and established that vancomycin co-precipitated with phosphate containing molecules. These findings highlight the high potential of this approach with nanometric spatial resolution which opens new perspectives for studies on drug-induced nephritis, nanocrystals and local lipid or glucid alterations.

Keywords
Renal biopsy, nanometric abnormal deposit, vancomycin, calcification, infrared nanospectroscopy.

Introduction

Generalization of kidney biopsy in the 1950's was the first step towards modern nephrology. Nowadays biopsy constitutes the gold standard for the diagnosis of kidney lesions.[1,2] Standard techniques allow precise diagnostic of most glomerular diseases.[3]

However, it is often difficult to infer from histological studies mechanisms of renal aggression in many cases of tubulointerstitial nephritis. Interestingly some of these diseases such as oxalosis or drug induced nephritis are characterized by the presence of abnormal deposits within the parenchyma. Chemical speciation of these compounds is of prime pathophysiological interest.

Physicochemical techniques such as Fourier Transform InfraRed (FTIR) or RAMAN microspectroscopies were developed for chemical mapping of the matter through light and matter interaction. FTIR spectroscopy is a common non-destructive physicochemical tool that allows collection of infrared absorption spectra and its integration into microscopy is commonly used to determine the chemical phase of kidney stones[4,5] and has contributed to the description of more than twenty types of abnormal deposits in kidney biopsies.[6–8] In 2017, using µFTIR, we identified the presence of vancomycin casts within the tubules of vancomycin exposed patients with acute kidney injury.[9] Nevertheless, due to its low spatial resolution, it was impossible to adequately study submicrometric crystals or deposits.

Here, we propose an innovative tool of infrared (IR) nanospectroscopy, called AFMIR[10–12], to overcome this barrier. This technique which is extensively applied in polymer science and microbiology and has recently been used for analysis of red blood cells, bone and skin tissue, allows chemical identification at the nanometer scale.[13–15] Its strength resides in the use of an atomic force microscope (AFM) to sense the IR absorption instead of classical optical devices commonly used in spectromicroscopes. The system acquires topographic images in contact mode. To perform IR measurements, the sample is highlighted with the IR laser (see details in the method section), an absorption phenomenon occurs inducing a rapid expansion (photothermal process) of the sample detected by the AFM tip. The signal acquired by the AFM is directly proportional to the IR absorbance.[10,16] Thus ultra-local IR spectra are acquired as well as IR maps.

For AFMIR, the xy spatial resolution is dependent on the AFM tip radius (around 10 nm) and the sensitivity along the z-axis is around 100 nm for the configuration used (see details in experimental section). Furthermore, besides its nanometric resolution, AFMIR provides large maps of the sample (90 µm) allowing to explore various compartments of human kidney biopsy.

We report the first chemical identification of abnormal deposits using this non-destructive IR nanospectroscopy in standard AFA-fixed paraffin-embedded kidney biopsy from patients with vancomycin cast nephropathy (described in [9]).

Experimental section

Kidney biopsy

Kidney biopsies were performed at Tenon Hospital (Paris, France). Patients description is provided in [9]. One-micron slices of the tissue samples were deposited on ZnSe prism. Paraffin was chemically removed with xylene. Samples were identified by a study number and anonymized. The ethical committee of Tenon Hospital had approved the patient consent procedure. For vancomycin immunochemistry: deparaffinized kidney sections were incubated for 30 minutes at 95°C in the target retrieval solution (Dako), blocked in PBS containing 5% BSA and immunostained against vancomycin (Abbot). After washing, immunostaining was revealed with Histofine secondary antibodies (Histofine) and then revealed with AEC (Dako). Nuclei were counterstained with hematoxylin.

Scanning electron microscopy

Observations of the microstructure were performed through a Zeiss SUPRA55-VP SEM. This field-emission "gun" microscope (FE-SEM) may operate at low voltage. High-resolution observations were obtained by using 2 secondary electron (SE) detectors: an in-lens SE detector and an Everharte-Thornley SE detector. Measurements were taken at low voltage without the usual deposits of carbon at the surface of the sample.

Conventional Infrared spectromicroscopy

IR μspectroscopy was performed in our hospital department in the "service des explorations fonctionnelles multidisciplinaires" (Tenon Hospital – Paris) on a spotlight 400 FTIR imaging system in the mid-infrared spectral range. Spectra were collected between 4000 and 700 cm^{-1}, each spectrum being acquired after 64 accumulations at 8cm^{-1} resolution.

Treatment of data provided by Conventional Infrared Spectromicroscopy

μFTIR spectra were smoothed at a resolution of 4 cm^{-1} by apodization of their Fourier transform by a Gaussian line, corrected for CO_2 with a flat line between 2450 and 2250 cm^{-1} and baseline corrected by straight lines interpolated between the spectra points at 3620, 3010, 2700, 2395, 2247, 1800, 1765, 1711 and 944 cm^{-1}. Images are composed of 26 pixels by 18, where each pixel covers an area of 6.25 μm by 6.25 μm. As there is area without sample, due to the sample preparation, the spectra are also filtered based on the signal to noise ratio. The signal is defined as the maximum intensities between 1760 and 1468 cm^{-1} and the noise is defined as the root mean square from 2000 to 1900 cm^{-1}. Area with a signal to noise < 100 are kept black on images. All treatment of μFTIR signal was carried out by Kinetics (SFMB, Brussels, Belgium).

Infrared nanospectroscopy

The AFMIR system is from Anasys instrument company. The configuration with the bottom-up illumination (total reflection) through a ZnSe prism was used. The IR pulsed laser source: an OPO produced 10 ns pulses sequentially tunable from 4000 to 1000 cm^{-1} with a repetition rate of 1kHz. Between 1600 and 1000 cm^{-1}, the diameter of the laser spot is around 50 μm. IR maps and spectra are normalized to the incident laser power. Local spectra were collected with a 2 cm^{-1} step scan and averaged on 256 pulses. The spectral resolution is estimated at 8 cm^{-1}. Here only the region between 1600 and 1010 cm^{-1} is displayed due to the IR source used for this study that can only performed spectral measurements sequentially (laser transition at 1599 cm^{-1}). Furthermore, after investigation no relevant differences were observed in the 1900 - 1600 cm^{-1} (amide I band) region. Topographic images were acquired in contact mode with silicon cantilevers of 0,03 N/m (μmasch HQ:CSC/AIBS).

AFMIR couples an atomic force microscope (AFM) with a tunable pulsed IR source to record spatially resolved IR absorption. Its principle is based on the detection of the photothermal effect induced after the absorption of the IR source, that leads to a local expansion. This expansion is detected thanks to the tip of the AFM directly in contact with the sample. A damped oscillation of the cantilever (ringdown) is observed. Previous studies have shown the oscillations amplitude is directly proportional to the absorbance.

During AFMIR measurements, lateral resolution is distinguished from axial sensitivity. Indeed, the axial sensitivity (smallest expansion detectable by the system) depends (among other) i) on the configuration used: classical configuration[18] or resonance-enhanced mode[19–21], ii) and also on the speed and the detection precision of the cantilever to track thermal expansion of the sample[22]. In our case, we used the classical configuration (ringdown of the cantilever) with standard cantilevers. As a consequence, our z-sensitivity is around 100 nm.

Other clarifications: the feasibility study was carried out upstream on intact renal biopsy. The section adheres well on the prism, its thickness is heterogeneous (0,2~3 μm). As it was shown that the response of the AFM-IR is linear between 0-1 μm thickness[16], several areas were probed for AFM-IR measurements to find the one with a thickness ≤ 1 μm. Ramer et al.

provided guidelines for selecting the sample parameters for best AFM-IR quantitative measurements[23].

Treatment of data provided by Infrared Nanospectroscopy

Topographic images and IR maps were analyzed using Mountainsmap 7.3 software (Digital Surf, France). IR spectra were averaged (2 spectra per points), filtered using Savitzky–Golay filter (second order, 10 pts) then normalized at 1530 cm^{-1}.

Results and discussion

Vancomycin casts are intratubular deposits forming hundred-nanometer spheres (nanospheres Figure 1A).[9] These casts can be spotted using bright field image (Figure 1B) to acquire IR microspectroscopy maps of the regions of interest and decipher chemical composition of the deposits (Figure 1 C-E). Standard protein content presents a strong absorption around 1650 cm^{-1} that allows global protein mapping (Figure 1C). Vancomycin can be distinguished from the other proteins and therefore mapped because it exhibits a global shift of the amide II band (Figure 1D). The IR spectra are rich in information as they contain the response of all cellular components. The absorption domain of the amide II band is in the 1600 to 1500 cm^{-1} range (mainly due to the N-H bending of the peptide bond). In normal tissue, this band is generally centered at around 1540 cm^{-1}(Figure 1E dark blue spectra) whereas it is centered at 1505 $cm^{-1}$for pure vancomycin (Figure 1E pure vancomycin reference spectra: black dotted curve). Note also that only vancomycin enriched spectra exhibit strong absorption bands around 1230 cm^{-1} and 1060-1020 cm^{-1}.

The spectra acquired along the yellow line drawn (Figure 1C) indicates a low to high vancomycin content depicted by a gradient of dark blue to red lines, respectively (Figure 1E). Although IR microspectroscopy is very specific, its spatial resolution is limited by diffraction and even large vancomycin casts do not exceed few micrometers. It is therefore not possible to finely probe deposits organization and composition.

The AFMIR system is equipped with standard optical microscopy view that allows standard histological identification (Supp. Fig. 1A). The AFM part of the device allows acquisition of topographic maps (Supp. Fig. 1B). Interestingly topographic maps are very similar to standard optical images and even closer to scanning electron microscopy (Supp. Fig. 1C).

We were able to acquire topographic maps of potential vancomycin containing casts in a first kidney biopsy (Figure 2A-B)[9]. We could then acquire IR maps with submicrometric resolution (Figure 2C). Vancomycin contribution to the composition of these nanospheres was confirmed with the acquisition of AFMIR spectra along a line from the border of the tubule to the deposit (yellow arrow Figure 2C, Figure 2D). A change of the spectra is observed, as we get closer to the deposit (dark blue to red gradient Figure 2D). These changes confirm the presence of vancomycin in the deposit, now at submicrometric resolution (compare Figures 2C and 2E-F).

Interestingly, even in the center of casts, the spectrum exhibits some discrepancies with the spectrum of pure vancomycin. A significant increase of the 1080 cm^{-1} band is observed. This region is generally associated with the symmetric stretching vibration of phosphates (PO_2^-), as observed in DNA, or with the finger print region for different polycarbonates. Such differences could be due to a complex aggregation process between cellular debris (protein, peptide, RNA, DNA, sugar…), vancomycin and/or products of vancomycin degradation. Interestingly possible interaction between vancomycin and RNA has been documented.[19]

Further identification of the nature of these unknown coprecipitates using mass spectrometry could help us deciphering vancomycin cast nephropathy physiopathology and improve its prevention.

Heterogeneity of vancomycin casts could be further illustrated by taking advantage of the unique ability of AFMIR to provide high-resolution topographic tissue maps. In a second case of vancomycin cast nephropathy, a suggestive deposit not only showed the usual spherical

intraluminal content (Figure 3A blue and green rectangles) but also a small squared shape crystal (Figure 3A red rectangle). Local IR spectra confirmed the presence of vancomycin mixed with organic debris and identified the crystal as calcium phosphate apatite (Figure 3 B) based on a massive absorption between 1200 and 1000 cm^{-1} characteristic of amorphous carbonated calcium phosphate (P-O band around 1060 cm^{-1}). Such deposit in the tubular lumen can be observed in healthy patients[17]

However, one could imagine that these inorganic formations could play a catalytic role in vancomycin cast formation. Since urinary alkali pH and urine concentration are the most common risk factor of urinary calcium phosphate apatite precipitation they should be monitored in prospective human and/or animal studies.

Conclusion

When abnormal deposits are found in kidney biopsies, it is of prime importance to determine their chemical composition in order to understand pathophysiology. Using AFMIR, we were able to identify vancomycin deposits at nanometric resolution. This super-high resolution allowed us to demonstrate that vancomycin casts are not only composed of pure vancomycin but contain phosphate rich organic material and that inorganic nanocrystals can be found within the lumen of the damaged tubules.

IR spectra are specific of vibration of the chemical bonds of the probed molecules. Therefore, IR is particularly adequate to identify exogenous non-biological molecules like drugs and inorganic material as their chemical formula strongly differs from standard tissue. Vancomycin being a peptide, it was challenging to isolate its spectra from the endogenous protein background. However, we were able to identify small modifications of classical amide bond (probably explained by the glycosylation and bacterial production of vancomycin). This shows the high potential of this approach that could be extrapolated to numerous applications ranging from drug-induced nephritis to nanocrystals and local lipids or carbohydrate alterations.

Acknowledgements

The authors would like to thank “la foundation du rein”: Prize for young researcher.

Supporting information paragraph

Figure to illustrate kidney morphology through topography. See Supporting Information for Publication file. One page and one figure.

Author information

The authors declare no financial competing interests.

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Graphics

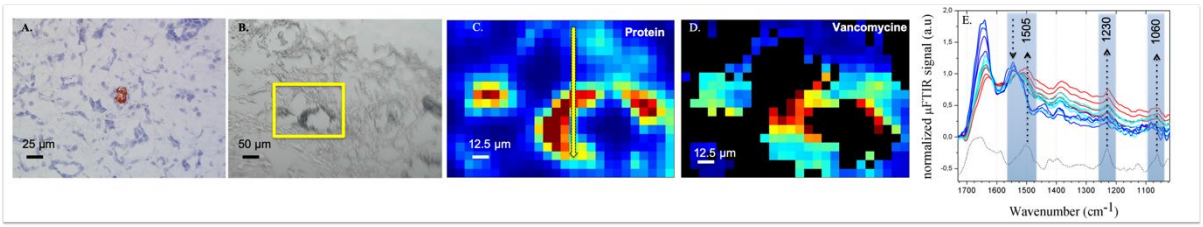

Figure 1: A. Standard immunochemistry reveals vancomycin casts in the form of intratubular nanospheres (anti-vancomycin antibodies); B. **microFTIR identification of vancomycin cast on a kidney biopsy:** phase contrast microscopy and C. chemical maps representing total protein content - absorbance at 1650 cm^{-1} or D. vancomycin - absorbance at 1505cm^{-1}, generated using normalized IR spectra of the area of interest (B yellow square). E. Normalized FTIR spectra acquired in each pixel of the image along the yellow line in (C) confirms the presence of vancomycin. Low and high vancomycin containing spectra are depicted in blue and red, respectively. Vancomycin reference spectrum is the black dotted line.

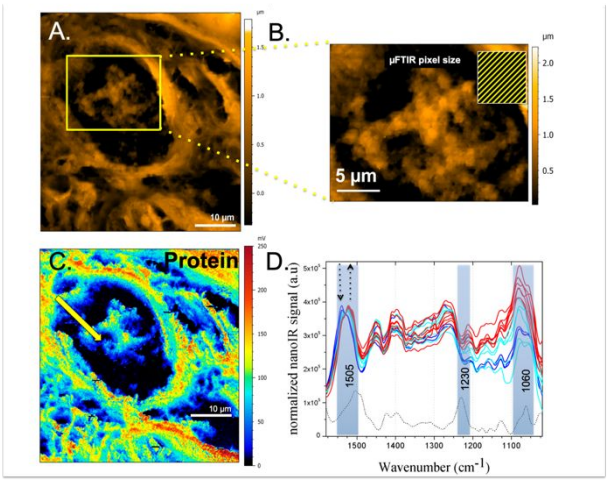

Figure 2: AFM-IR Analysis: A. and B. Nanospheres evocative of vancomycin cast can be identified on kidney biopsy using AFM topographic maps. C. Highly spatially resolved chemical mapping (protein content 1650 cm^{-1}) and local IR spectra within the vancomycin suggestive cast (yellow arrow C, local spectra presented in D).
In D, these spectra show the IR absorption bands assigned to vancomycin (black arrows) and also contained phosphate rich organic material. There is a vancomycin gradient (dark blue to red spectra) between center and periphery of the deposit. In the very center of the cast spectra exhibit some discrepancies with the spectrum of pure vancomycin (black dotted line) indicating co-precipitation.

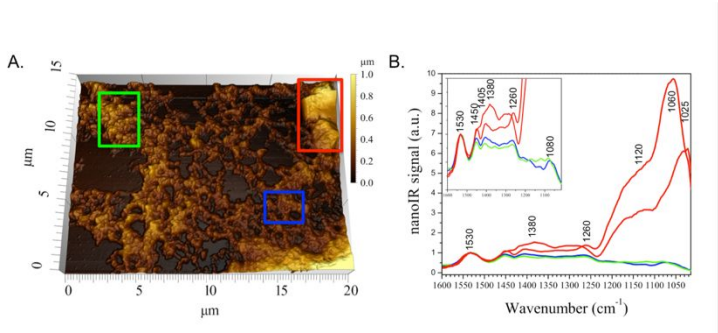

Figure 3: Heterogeneities in vancomycin casts: A. Topographic image evidences two different textures. The deposit is probably a mix of drug accumulation mixed with organic material (green and blue rectangles) and calcification (upper left corner - red rectangle). B. Local IR spectra confirmed presence of vancomycin mixed with cellular debris and a calcium phosphate apatite nanocrystal (huge absorption band 1200 - 1000 cm^{-1} red spectra).

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