Research Article

Improvement of Urinary Stones Analysis Combining Morphological Analysis and Infrared Spectroscopy

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Daudon et al. have developed a complex morphoconstitutional classification of renal stone in six different morphological types and several subtypes. According to this classification, a precise correspondence exists between causes of renal stones and subtypes with a great clinical relevance and can be considering a sort of shortcut for the metabolic diagnosis in renal stone patients. Now the diagnosis of causes of renal stones generally requires repeated biochemical investigations on urine and blood samples and usually remains presumptive. We analyzed 150 urinary stones both by stereoscopic microscopy and Fourier transform infrared spectroscopy. The comparison of 150 stones did not reveal any disagreement. We have only 20 partial agreement, and clinicians agreed that the imprecise information obtained with morphological analysis alone would have missed an important clinical finding only in 3 cases. In conclusion, in our opinion, the analysis of urinary stone must combine two different analytical techniques: morphological analysis by stereomicroscope and biochemical analysis with the FT-IR.

1. Introduction

Daudon et al. have developed a complex morphoconstitutional classification of renal stones which gives important hints on the pathogenesis of renal stones [1]. This classification is used by other laboratories [2–4]. Six different morphological types of renal stones are identified with several subtypes (Table 1). The crystalline components correspond to specific subtypes, although different subtypes may have the same crystalline component. The associations between different subtypes are also commonly observed in stones, and six main associations have been described by Daudon et al. [1]. According to this classification, a precise correspondence exists between causes of renal stones and subtypes. It is for this correspondence that such a classification would have huge clinical relevance since it would

constitute a sort of shortcut in the metabolic diagnosis in renal stone patients. Moreover, it would be a perfect window on the pathogenesis of that specific stone which, otherwise, frequently remains vague. Indeed, now, the diagnosis of causes of renal stones generally requires a number of biochemical investigations on urine and blood samples. Furthermore, due to the variability of many of the parameters, especially those determined in urines, repeated determinations are advocated [5]. Except for the secondary forms of calcium renal stones and for some rare noncalcium stones, the diagnosis of the metabolic causes of a stone remains presumptive. In fact, very rarely, we grasp the metabolic phenomenon determining lithogenesis and stone growth in the precise moment when these phenomena are going on. More frequently, we see some phenomenon occurring months later or earlier of a stone episode. Saying

TABLE 1: Morphocostitutional classification of renal stones developed by Daudon et al.

Morphological type	Subtype	Main components	Common causes
I	Ia	Whewellite	Dietary hyperoxaluria
	Ib	Whewellite	Stasis, low diuresis
	Ic	Whewellite	Primary hyperoxaluria type I
	Id	Whewellite	Malformative uropathy, stasis and confined multiple stones
	Ie	Whewellite	Enteric hyperoxaluria
II	IIa	Weddellite	Hypercalciuria
	IIb	Weddellite ± whewellite	Hypercalciuria ± hyperoxaluria ± hypocitraturia
	IIc	Weddellite	Hypercalciuria, stasis and confined multiple stones
III	IIIa	Uric acids	Low urine pH and stasis
	IIIb	Uric acids	Metabolic syndrome, diabetes
	IIIc	Various urates	Hyperuricosuria and alkaline urine, UTI
	IIId	Ammonium urate	Hyperuricosuria and diarrhea
IV	IVa1	Carbapatite	Hypercalciuria, UTI
	IVa2	Carbapatite	Distal renal tubular acidosis
	IVb	Carbapatite	UTI, hypercalciuria
	IVc	Struvite	UTI by urease-splitting bacteria
	IVd	Brushite	Hypercalciuria, PHPT, phosphate leak
V	Va	Cystine	Cystinuria
	Vb	Cystine	Cystinuria + inadequate therapy
VI	VIa	Proteins	Chronic pyelonephritis
	VIb	Proteins	Proteinuria, drugs, clots
	VIc	Proteins	ESRF and excessive calcium + vitamin D supplementation

with CL Smith's words, since "*Stones recovered from the urinary tract are like rocks to the archeologist in that their study discloses information regarding their formation*" [6], if we are able to classify this information, then we would have a strong diagnostic tool in our hands. Daudon's classification is at the moment the strongest attempt to reach this result.

Grinding the sample for IR analysis is still the main inconvenience because of the loss of spatial distribution of the different minority compounds present in the kidney stone. The location of major and minor components in the stone is of major importance; in fact, the precise point of the stone where each component precipitates is generally linked to the urine conditions at the time of the corresponding solid formation. The metabolic changes whose patients suffer from remain imprinted in the stone, defining a particular distribution of the components. The morphological analysis with stereoscopic microscopy is a technique which gives complementary information to the IR analysis.

For these reason, we would like to introduce, in our laboratory, morphological analysis (MA) to be used together with the IR technology already introduced in routine since 2013 [7].

The aim of our paper was to analyze 150 urinary stones both with infrared spectroscopy and stereoscopic microscopy to underline the usefulness to introduce the analytical methodology known as morphocostitutional analysis and improve the accuracy of urinary stone classification.

2. Materials and Methods

We analyzed 150 consecutive urinary stones, from 150 patients (68% men and 32% women; age range 29–82) from the Divisions of Nephrology and Urology of our University Hospital.

Stones were washed with deionized water and dried at room temperature for 24 h. After that, for each stone, shape, color, size, and weight were registered. Stones were then analyzed both by stereoscopic microscopy (STEM) and Fourier transform infrared spectroscopy (FT-IR) as described below. When a discordance occurred (less than 10% of cases), a joint evaluation was performed to share the allocation of cases. This was possible in all discordant cases.

Finally, the clinicians were separately interrogated on how the discrepant results would have modified their diagnostic and therapeutic conclusions.

2.1. FT-IR Analysis. For FT-IR analysis, stones were powdered and mixed with an inert powdered support (dried potassium bromide) in a proportion of 0.5 to 2% in agate mortar. This mixture was transferred into an appropriate die and pressed at $10 \, \text{t/cm}^2$ to form a transparent pellet 13 mm in diameter. The pellet assembled in a holder was placed in the infrared (IR) beam of the spectrometer. The spectral region investigated was from 4000 to $400 \, \text{cm}^{-1}$; 32 scans were averaged with a $4 \, \text{cm}^{-1}$ resolution for each spectrum. Background spectra were performed at air or pure KBr pellet before each analysis (sample blank). Spectra were recorded by means of a PerkinElmer Spectrum One [8]. Spectra were then computer-matched with the Euclidean search application, a tool of SPECTRA NICODOM IR Library (obtained from Nicodom s.r.o., Hlavni 2727 CZ-14100 Praha 4, Czech Republic, EU) that compares the unknown spectrum with reference spectra contained in the library between 4000 and $400 \, \text{cm}^{-1}$. A report is then generated for the various stone components. The results of the automatic comparison for

TABLE 2: Composition and agreement in the pure stone group.

Substances detected (n)	Pure stones		
	Agreement (n)	Partial agreement (n)	Disagreement (n)
Calcium oxalate monohydrate (47)	44	3	0
Calcium oxalate dihydrate (1)	1	0	0
Carboapatite (1)	1	0	0
Anhydrous uric acid (4)	4	0	0
Brushite (2)	2	0	0
Ammonium urate (1)	1	0	0
Cystine (10)	10	0	0
Proteins (5)	5	0	0
Total (71)	68 (95.7%)	3 (4.3%)	0 (0.0%)

spectrum identification were provided as a list of the best-fitting spectra with their scores. The score value can range from 0.000 to 1.000. Score 1.000 indicates a perfect likeness between the unknown spectrum and the reference one. In each case, a visual inspection of the spectra was performed to check the results and assign the composition.

2.2. Morphological Analysis. MA was conducted in accordance with the protocol published by Daudon et al. [1] (Table 1). The sample analyzed by STEM was observed at different magnifications (from 7.5x up to 50x). After surface observation and identification of subtypes, the stone was sectioned and the identification of subtypes in the core, middle, and outer layers was performed separately. In the case of stones obtained after extracorporeal shock wave lithotripsy (ESWL), all available fragments were studied to determine form and structure. Stones containing two or more components were classified as "mixed stones", and their major and minor components were identified. The cutoff between minor and major components was specified as <40%. The cutoff for elements in trace was <5%.

2.3. Method Comparison. On the basis of FT-IR analysis, calculi were divided into three groups: pure stones ($n = 71$), mixed stones ($n = 68$), and pure stones with components in trace ($n = 11$). Results of each group were compared with those obtained with MA.

Classification criteria were established by comparing results obtained with the two methods in reference to the identification of different species as follows:

(i) *Agreement*: when FT-IR and MA identified the same components

(ii) *Partial agreement*: when MA identified the main stone component(s) detected by FT-IR but in addition there were some disagreement for secondary constituents

(iii) *Disagreement*: when MA did not identify the main stone component

3. Results

The analysis of 150 stones did not reveal any disagreement. In Tables 2–4, the comparison between the two methods is

reported for pure, pure with substances in trace, and mixed stones, respectively. The large agreement in all groups is clearly evident; in fact, only 4.2%, 18.1%, and 22.1% were in partial agreements among pure stones, pure stones with substances in trace, and mixed stones, respectively.

Seventy-one were the pure stones, that is, composed of just one crystalline species (Table 2). The most represented type was calcium oxalate (CaOx) as monohydrate crystal (COM). In this category, we observed 3 discordant cases (#13, #18, and #60); indeed, MA suggested the presence of dihydrate CaOx (COD) not detected by FT-IR in all three cases. However, this discrepancy could be explained by an intermediate transition form of COD to COM on the stone surface. Thus, there is a reason to consider this finding as congruent with the FT-IR analysis.

In 11 stones, the FT-IR disclosed the presence of a second crystalline species in trace, which was not discovered by the MA in 2 cases (#84 and #119) (Table 3). In both, traces of carboapatite were below the cutoff limit for substances in trace.

Sixty-eight were the mixed stones examined, that is, 2 or more components sometimes associated with further components in traces (Table 4). Full agreement between FT-IR and MA was observed in 53. The examination of the remaining 20 stones gave discordant results (Table 5). In particular, in #29, #67, and #87, the FT-IR spectroscopy did not identify the COD component which was recognized by the MA; this happened always on the MA of the surface of the stone where the transition of COD to COM was described. Another significant discrepancy occurred in two stones (#96 and #100) where dihydrate uric acid crystals were observed by MA at odds of FT-IR which reported only anhydrous crystals.

A number of other cases were discordant for the presence of tiny quantities of some crystalline component which was not identified by MA.

In a CaOx stone (#42 and #90) (COM/COD 70/30% and 85/10%, resp.), the small quantity (<5%) of carboapatite was not recognized. Of note, the examined specimen was a fragment of a stone recovered after shock wave lithotripsy.

In specimens #12 and #75, both containing a significant quantity of carboapatite (60 and 10%, resp.), a tiny quantity below the cutoff limit (<5%) of brushite and/or octacalcium phosphate was also identified by FT-IR spectroscopy. Both were stone fragments obtained after ESWL and ureteroscopy, and ureteroscopy, respectively.

Table 3: Composition and agreement in pure stone with substances in trace group.

Substances detected (n)	Pure stones with substances in trace		
	Agreement (n)	Partial agreement (n)	Disagreement (n)
Anhydrous uric acid + calcium oxalate monohydrate trace (1)	1	0	0
Calcium oxalate monohydrate + calcium oxalate dihydrate trace (1)	1	0	0
Carboapatite + struvite (1)	1	0	0
Cystine + carboapatite (1)	1	0	0
Protein + carboapatite trace (1)	1	0	0
Struvite + carboapatite trace (1)	0	1	0
Brushite + carboapatite trace (1)	1	0	0
Brushite + calcium oxalate monohydrate trace (2)	2	0	0
Calcium oxalate monohydrate + carboapatite trace (2)	1	1	0
Total (11)	9 (81.8%)	2 (18.2%)	0 (0.0%)

Table 4: Composition and agreement in the mixed stones group.

Substances detected (n)	Mixed stones		
	Agreement (n)	Partial agreement (n)	Disagreement (n)
Calcium oxalate monohydrate + calcium oxalate dihydrate (9)	9	0	0
Anhydrous uric acid + calcium oxalate monohydrate (6)	4	2	0
Calcium oxalate monohydrate + calcium oxalate dihydrate + carboapatite (13)	11	2	0
Anhydrous uric acid + dihydrate uric acid + calcium oxalate monohydrate (2)	1	1	0
Calcium oxalate monohydrate + calcium oxalate dihydrate + carboapatite + brushite + octacalcium phosphate (1)	0	1	0
Carboapatite + calcium oxalate monohydrate + Whitlockite (1)	0	1	0
Protein + calcium oxalate monohydrate (1)	1	0	0
Cystine + carboapatite (3)	3	0	0
Carboapatite + struvite + calcium oxalate monohydrate (1)	0	1	0
Carboapatite + struvite (10)	9	1	0
Carboapatite + struvite +brushite (1)	1	0	0
Carboapatite + struvite + ammonium urate (1)	1	0	0
Brushite + calcium oxalate dihydrate (1)	1	0	0
Anhydrous uric acid + ammonium urate + calcium oxalate monohydrate (1)	0	1	0
Anhydrous uric acid + ammonium urate + struvite + calcium oxalate monohydrate + sodium urate (1)	1	0	0
Ammonium urate + anhydrous uric acid (1)	1	0	0
Ammonium urate + carboapatite (1)	1	0	0
Calcium oxalate dihydrate + carboapatite (2)	2	0	0
Carboapatite + magnesium ammonium phosphate (2)	2	0	0
Carboapatite + protein + triglycerides (1)	0	1	0
Carboapatite calcium oxalate dihydrate + brushite (1)	0	1	0
Calcium oxalate dihydrate + brushite (1)	1	0	0
Calcium oxalate monohydrate + carboapatite (7)	4	3	0
Total (68)	53 (77.9%)	15 (22.1%)	0 (0.0%)

Tiny quantities of COM were also missed by MA in 2 uric acid-containing stones (#26 and #128) and in a mixed carboapatite and struvite stone (#130).

Conclusions for stone #5 were discordant because FT-IR recognized protein and triglyceride components (15% and <5%) in these quantities cannot be recognized on MA. The

Table 5: Composition for the 20 discordant results observed by MA and FT-IR.

#Stone	MA			FT-IR											
	Core	Section	Surface	COM	COD	CARBA	STRU	BRU	AU0	AU1	AU2	AmUr	PROT	OCP	WK
5		IVa1				85							15		
12	IVa1	IVa1 + IIa	IVa1		40	60		<5							
13		Ib	IIb--> Ia	100											
18	Ia	Ia	IIb--> Ia	100											
26		IIIa	IIIb	<5					50		50				
29		Ia + IVa	IIb--> Ia	95		5									
42		Ia	IIb--> Ia	70	30	<5									
60		Ia	IIb--> Ia	100											
67		Ia + IVa	IIb--> Ia	90		10									
75		Ia + IVa	IIa	70	20	10		<5						<5	
81	IVa1	Ia + IVa1	IVa1	10		70									20
84		Ia	Ia active	100		<5									
87		Ia + IVa	IIb--> Ia	95		5									
90		Ia	IIb--> Ia	85	10	5									
96	Ia	Ia + IIIb	IIIb	50					50						
100		Ia	IIIb	90					10						
119		IVc	IVc			<5	100								
128	IIIc	IIIa		<5					70				30		
130		IVa + IVc	IVa + IVc	<5		30	70								
134		IVc	IVc			10	90								

COM, monohydrate calcium oxalate; COD, dihydrate calcium oxalate; CARBA, carboapatite; STRU, struvite; BRU, brushite; AU0, uric acid anhydrous; AU1, uric acid monohydrate; AU2, uric acid dehydrate; AmUr, ammonium urate; PROT, protein; OCP, octacalcium phosphate; WK, Whitlockite; IIb--> Ia indicates the transition phase COD to COM in the stone.

analysis of stone #81 gave discrepant results because it was composed by carboapatite (70%), COM (10%), and Whitlockite (20%), a calcium phosphate crystal rarely observed in renal stones, that cannot be recognized at MA. Finally, in stone #134, whose main component was struvite, carboapatite (10%) was not recognized.

4. Discussion

In this study, we compared the results obtained for 150 urinary stones analyzed using both the FT-IR and stereomicroscopy. As expected, this study confirms the concordance of the morphological subtypes of stones and the crystalline components. In fact, this was observed in 87 to 94% of cases depending on the conservativeness of the criteria used.

According to the morphoconstitutional classification of Daudon et al. [1], COM is generally associated with hyperoxaluric states, while COD suggests the existence of hypercalciuria. However, since the conversion from COD to the more stable COM crystalline phase is a well-known phenomenon [9], some derangement from this strict dichotomy is expected. In the eight stones which were discordant (the case of #13, #18, #29, #42, #60, #67, #87, and #90 (Table 5)), the disagreement was only observed on the surface of the stone that was most recently formed. Interestingly, the appearance was that of the subtype IIb, that is, a mixture of COD and COM. In this condition, when COD is almost completely converted to COM, the FT-IR signals are difficult to be interpreted and the morphological investigation of the stone for a conclusive definition of the crystalline species is necessary. Not recognizing the COD component in a stone would in theory have stronger clinical implications because of neglecting the frequent association with hypercalciuria. However, in this case, it was the FT-IR not the MA that gave incomplete information, failing to recognize that a certain portion of CaOx existed in the transition phase COD to COM in the stone.

In two pure stones where FT-IR disclosed trace elements of a second crystalline species (#84 and #119; Table 5), MA did not recognize the constituent in trace. Interestingly, stone #84 was indeed a post-ESWL fragment, and the unrecognized component was carboapatite. The existence in otherwise pure stones of COM of minor quantities, generally less than 10%, of carboapatite is typical of the "idiopathic" CaOx nephrolithiasis and considered to be a remnant of Randall's plaque on which the stone formed [10]. This once more advises for collecting as much as possible fragments of a stone to perform the analysis of its components. In fact, properly recognizing Randall's plaque residue in a CaOx stone gives very important and useful pathogenic and diagnostic clues [11]. However, this is not accepted by all, and thus, the idiopathic nature of the CaOx nephrolithiasis still requires a full diagnostic workup [12].

The second case (#119) was a stone made of struvite, a structure which forms in an alkaline milieu that also favors carboapatite crystallization.

In mixed stones (Table 4), the presence of minute quantities of brushite (<5%) in stones containing carboapatite (#12 and #75) is also not unexpected since brushite is considered to be a crystalline precursor of the latter, a clinically useless information [13]. The same for uric acid containing stones #26 and #128 which had COM in trace. The presence of CaOx in a uric acid stone is predicted by the

promoting role of uric acid on CaOx crystallization [14]. Finally, the minute percentage of COM in uric acid containing stones (#26 and #128) would not change the clinical approach since preventive treatment of uric acid stones certainly does not aggravate the risk of CaOx lithogenesis.

In our opinion, all stones disclosing at FT-IR spectroscopy tiny quantities (<5%) of some crystalline components should not be considered as truly discordant if that component is not described at MA. In fact, not recognizing under the STEM such a tiny quantity of COM is quite comprehensible because probably it existed in a very focal, microscopic location in the stone.

Main discordances were observed in the 2 stones primarily composed by carboapatite, stone #5 and #81, in which protein or Whitlockite were recognized, respectively. The stereomicroscopic examination does not consent to identify proteins in a stone if it is scantily represented. It can identify Whitlockite due to the lack of a specific morphology for this rare calcium phosphate component of renal stones. Whitlockite, a calcium magnesium orthophosphate $(Ca, Mg)_3 (PO_4)_2$, is generally found in women with stones associated with chronic urinary tract infections by nonurease-producing bacteria [15]. Proteins are a common constituent of kidney stones, but at marginal concentrations, that is, less than 2.5% dry weight [16, 17]. On the contrary, proteins are much more represented in stones associated with chronic urinary infections as likely in this case. Thus, these 2 cases suggest a role of urinary tract infections in their pathogenesis. The condition of the struvite stone #134 where MA did not reveal 10% carboapatite could be similar. While the presence of carboapatite could denote the metabolic origin of the stone followed by a super infection causing struvite precipitation [18], an entire infectious origin cannot be discarded. A more in-depth analysis with the determination of the carbonation rate of carboapatite in these stones would support this role [19].

What would be the consequence of the "wrong" diagnosis of stone composition with MA? Finding a relevant quantity of brushite in a stone is an important clinical observation due to the specific profile of patients forming brushite stones, that is, recurrent stone formers who had multiple previous ESWL and who may have converted from another stone composition [13, 20]. These patients have hypercalciuria but not distal renal tubular acidosis and primary hyperparathyroidism [13]. Furthermore, they may have obstruction of the urinary tract, either congenital or acquired [21]. Even the main discordance observed in stones #96 and #100 where dihydrate uric acid crystals were observed by MA at odds of anhydrous uric acid recognized by FT-IR has a minor if not irrelevant clinical meaning. Actually, both crystalline forms are due to conditions characterized by low urine pH [22].

The microscopic analysis of composition of different areas (core, middle layer, and surface) of urinary stone permit to understand urinary stone genesis and to know the origin of this event allowing, together to FT-IR analysis, a more targeted clinical intervention for reducing the risk of recurrence.

According to morphological characterization of Daudon, COM urinary stones are divided into five subtypes (Ia, Ib, Ic, Id, and Ie), each of them associated with different etiologies [1], and then, the identification of specific subtype, not possible with IR analysis, helps to reach the correct diagnosis. For instance, we have identified some renal stones as subtype Ie with STEM and pure COM with FT-IR. The subtype Ie is associated with enteric hyperoxaluria and in all these cases clinicians have confirmed inflammatory bowel diseases (IBD) for these patients. It is clear that an uncommon subtype like Ie can be very useful for the clinician in order to diagnose a severe cause of hyperoxaluria, often responsible of chronic kidney disease and end-stage kidney disease (ESKD) [23] and not always previously identified with other diagnostic investigations.

Another example that underlines the importance of morphological analysis can be seen in calcium phosphate kidney stones, which constitutes a very heterogeneous group and are divided into five morphological subtypes (IVa1 and IVa2 and IVb, IVc, and IVd) each of them associated with multiple etiologies, including urinary tract infection and/or different metabolic disorders.

As it was reported in the study carried out by Daudon et al. [24], infrared spectra of the subtypes IVa1 and IVa2, both composed of carbonate apatite $(Ca_5(PO_4)_3OH)$, are similar, but the two subtypes have different morphological structures. The first subtype (IVa1) has a homogeneous rough surface and a poorly organized section of diffuse. The second subtype (IVa2) shows a peculiar morphology characterized by smooth surface with glazed appearance and small cracks.

Type IVa1 is observed in case of urinary tract infection (UTI) and in patients with idiopathic hypercalciuria, while type IVa2 was associated with 90% all cases with distal renal tubular acidosis (dRTA).

Indeed, in our study, for a stone type IVa2, the clinicians have confirmed the diagnosis made with STEM analysis. It is obvious that such detailed information can be very helpful in addressing clinical diagnosis.

FT-IR technique shows a high sensitivity and allows an accurate identification of stone composition, but it cannot discriminate into different subtypes. The advantage of adding a stone morphology examination to infrared analysis is to provide a rapid, inexpensive orientation to specific pathological conditions that could not be readily identified when extensive metabolic investigations are not systematically performed.

Renal stone analysis should be able to identify, in a standardized way, all the characteristics that show a correlation with the etiopathogenesis of the disease: chemical composition, crystalline species, morphological characteristics, the sequence of precipitation of the components, and relative amounts of each of them. No analytical technique taken individually is able to provide all these information. For these reasons, a specialized center in the analysis of urinary stone must combine two different analytical techniques: morphological analysis by stereomicroscope, to determine the morphological characteristics and the precipitation sequence of components, and biochemical analysis with the FT-IR, for the determination and quantification of the individual components.

Ethical Approval

No animals were included in the study. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.
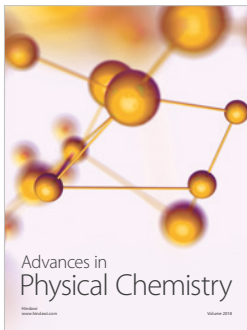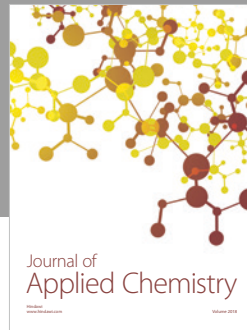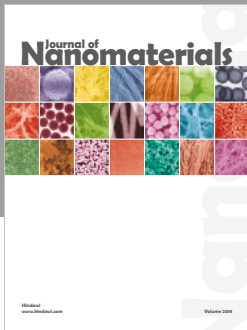
Disclosure

Aniello Primiano is the co-first author.

Conflicts of Interest

None of the authors have any potential conflicts of interest.

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