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TITLE:

Estimation of urinary nanocrystals in humans using calcium fluorophore labeling and Nanoparticle Tracking Analysis

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KEYWORDS:

Oxalate, kidney stones, nanocrystalluria, Nanoparticle Tracking Analysis, calcium

SUMMARY:

The objective of this study was to determine whether nanoparticle tracking analysis (NTA) could detect and quantify urinary calcium containing nanocrystals from healthy adults. The findings from the current study suggest NTA could be a potential tool to estimate urinary nanocrystals during kidney stone disease.

ABSTRACT:

Kidney stones are becoming more prevalent worldwide in adults and children. The most common type of kidney stone is comprised of calcium oxalate (CaOx) crystals. Crystalluria occurs when urine becomes supersaturated with minerals (e.g., calcium, oxalate, phosphate) and precedes kidney stone formation. Standard methods to assess crystalluria in stone formers include microscopy, filtration, and centrifugation. However, these methods primarily detect microcrystals and not nanocrystals. Nanocrystals have been suggested to be more harmful to kidney epithelial cells than microcrystals in vitro. Here, we describe the ability of Nanoparticle Tracking analysis (NTA) to detect human urinary nanocrystals. Healthy adults were fed a controlled oxalate diet prior to drinking an oxalate load to stimulate urinary nanocrystals. Urine was collected for 24 hours before and after the oxalate load. Samples were processed and washed with ethanol to purify samples. Urinary nanocrystals were stained with the calcium binding fluorophore, Fluo-4 AM. After staining, the size and count of nanocrystals were determined using NTA. The findings from this study show NTA can efficiently detect nanocrystalluria in healthy adults. These findings suggest NTA could be a valuable early detection method of nanocrystalluria in patients with kidney stone disease.

INTRODUCTION:

Urinary crystals form when urine becomes supersaturated with minerals. This can occur in healthy individuals but is more common in individuals with kidney stones[1]. The presence and

accumulation of urinary crystals can increase one's risk of developing a kidney stone. Specifically, this occurs when crystals bind to Randall's plaque, nucleate, accumulate, and grow over time[2-4]. Crystalluria precedes kidney stone formation and assessment of crystalluria may have predictive value in kidney stone formers[3,5]. Specifically, crystalluria has been suggested to be useful to predict the risk of stone recurrence in patients with a history of calcium oxalate containing stones[6,7].

Crystals have been reported to negatively impact renal epithelial and circulating immune cell function[8-13]. It has been previously reported that circulating monocytes from calcium oxalate (CaOx) kidney stone formers have suppressed cellular bioenergetics compared to healthy individuals[14]. In addition, CaOx crystals reduce cellular bioenergetics and disrupt redox homeostasis in monocytes[8]. Consumption of meals rich in oxalate may cause crystalluria which could lead to renal tubule damage and alter the production and function of urinary macromolecules that are protective against kidney stone formation[15,16]. Several studies have demonstrated that urinary crystals can vary in shape and size depending on the pH and temperature of the urine[17-19]. Further, urinary proteins have been shown to modulate crystal behavior[20]. Daudon et al.[19], proposed that crystalluria analysis could be helpful in the management of patients with kidney stone disease and in assessing their response to therapies. A few conventional methods currently available to evaluate the presence of crystals include polarized microscopy[21,22], electron microscopy[23], particle counters[3], urine filtration[24], evaporation[3,5] or centrifugation[21]. These studies have provided valuable insight to the kidney stone field regarding crystalluria. However, a limitation of these methods has been the inability to visualize and quantify crystals less than 1 μm in size. Crystals of this size may influence the growth of CaOx stones by attaching to Randall's plaque.

Nanocrystals have been shown to cause extensive injury to renal cells compared to larger microcrystals[25]. The presence of nanocrystals has been reported in urine using a nanoparticle analyzer[26,27]. Recent studies have used fluorescently labeled bisphosphate probes (alendronate-fluorescein/alendronate-Cy5) to examine nanocrystals using nanoscale flow cytometry[28]. The limitation of this dye is that it is not specific and will bind to almost all types of stones except cysteine. Thus, accurately assessing the presence of nanocrystals in individuals may be an effective tool to diagnose crystalluria and/or predict stone risk. The purpose of this study was to detect and quantify calcium containing nanocrystals (<1 μm in size) using nanoparticle tracking analysis (NTA). To achieve this, NTA technology was used in combination with a calcium binding fluorophore, Fluo-4 AM to detect and quantify calcium containing nanocrystals in the urine of healthy adults.

PROTOCOL:

All experiments outlined in this work were approved by the University of Alabama at Birmingham (UAB) Institutional Review Board. Healthy adults (33.6 ± 3.3 years old; n=10) were enrolled in the study if they had a normal blood comprehensive metabolic panel, non-tobacco users, non-pregnant, a BMI between 20-30 kg/m^2, and free of chronic medical conditions or acute illnesses. Healthy participants signed a written informed consent form prior to the start of the study.

1. Clinical protocol and urine collection

1.1. Have participants consume a low oxalate diet prepared by the UAB Center for Clinical and Translational Sciences Bionutrition Core for 3 days and fast overnight before collecting their urine (24-hour sample).

1.2. The following day, have participants return their 24-hour urine sample (pre-oxalate) before consuming an oxalate load (smoothie containing fruits and vegetables, ~8 mM oxalate). Have participants subsequently collect their urine for 24 hours (post-oxalate sample) and return their urine the following day.

1.3. Maintain all urine samples at room temperature (RT) prior to processing as described below and shown in **Figure 1**.

2. Urine Processing

NOTE: All materials and equipment used are listed in **Table of Materials**.

CAUTION: Wear personal protective equipment at all times while handling clinical samples and reagents. Specifically, gloves, face and eye shields, respiratory protection, and protective clothing.

2.1 Measure and record urine pH and volume. Mix thoroughly prior to adding 50 mL of urine into a labeled sterile 50 mL conical tube.

2.2 Centrifuge sample at 1200 x *g* for 10 min at RT using a benchtop centrifuge.

NOTE: Keep the sample at RT to prevent further crystal formation as cooler temperatures can promote crystallization.

2.3 Discard the supernatant and wash and resuspend the pellet again with 5 mL of 100% ethanol. Centrifuge the sample at 1200 x *g* for 10 minutes at RT using a benchtop centrifuge.

2.4 Discard the supernatant and resuspend the pellet in 1 mL of 100% ethanol. Store the sample at -20 °C for later processing OR stain the sample as described below.

NOTE: There is no significant difference in data points (i.e., particle size/concentration) between stored or freshly stained samples.

3. Nanoparticle Tracking Analysis (NTA)

3.1 Sample Preparation

3.1.1 Gold nanoparticles: Use gold nanoparticles to optimize settings on the instrument. Dilute 100 nm sized gold nanoparticles 1:1000 in ultrapure water.

3.1.2 Human Urine: Dilute urine samples 20 times in water prior to staining with 5 mM Fluo-4 AM (a calcium fluorescence dye) for 30 min in the dark. Analyze the samples using NTA.

3.1.3 Prepare Calcium Oxalate (CaOx) crystals as previously described[29]. Dilute 10 mM stock solution (14.6 mg in 10 mL of water) to 50 µM in water and stain the diluted samples using 5 mM Fluo-4 AM for 30 min in dark prior to analysis.

3.1.4 Calcium Phosphate (CaP) crystals: Dilute 10 mM stock solution (50.4 mg in 10 mL of water) to 50 µM in water and stain the diluted samples using 5 mM Fluo-4 AM for 30 min in dark prior to NTA analysis.

3.2 Instrument Set-up, Camera Settings, and Data Collection

NOTE: The computer and instrument setup used for this method are shown in **Figure 2**.

3.2.1 Turn on the computer and then the instrument. Open the software and turn on the camera.

3.2.2 Once the software window is open, click the capture icon in the top left corner on the window to start the capture mode. The camera initialization takes a few seconds.

3.2.3 Clean the platform by first pumping air into it using a 1 mL syringe until the platform appears clean. Gently add water to the apparatus 2-3 times using another 1 mL syringe to remove any air bubbles.

NOTE: Look for any air bubbles in the platform as well as in the tubing. It is important to not have bubbles throughout the apparatus prior to and while running samples. If bubbles are present, clean the platform again with air and water.

3.2.4 Once the platform is clean, add water to check for any contamination on the surface by viewing the camera. Once the platform is clean, add gold nanoparticles (1 mL, 100 nm; 3.8×10^8 particles/mL) as a control to the sample loading pump injector to set up the instrument.

3.2.5 Adjust the camera level on the screen or on the knob to the right side of the instrument until the image starts to display colored pixels and then reduce the camera level.

3.2.6 Then adjust the screen to optimize the image. **Left-click** the mouse button on the video image. **Hold** the **left mouse button** and **drag** image up and down to get the entire view.

NOTE: The normal camera lens and filter is used to assess gold nanoparticles and unstained samples.

3.2.7 Set up the infusion speed and focus the camera so that the gold nanoparticles are visible on the camera screen. Set the infusion speed to high (i.e., 500 µL/min) for initial set up to ensure the gold nanoparticles are detected. Once detected, reduce the speed to 50 µL/min to visualize gold nanoparticles.

3.2.8 Adjust the camera level to visualize the particles. For unstained samples, adjust the screen gain at level 5 to achieve the camera focus, and set the camera level at 8. Once the focus is set, record the sample (i.e., 1 measurement for 60 seconds only).

NOTE: The focus and continuous flow speed are important for obtaining clear and sharp images of the particles for counting.

3.2.9 After optimization, clean the apparatus again with water before assessing samples. View the camera to ensure that the tubing is clean and particles are not present.

NOTE: Wash the chamber between each sample until no particles are detected by the camera.

3.2.10 To analyze stained samples, adjust the camera to the filter position containing the suitable fluorescent filter. Load diluted and stained samples onto the sample loading pump injector and reduce the speed to 20 µL/min for analysis of the sample.

3.2.11 Next adjust the screen gain and camera level as these are important parameters. For stained (fluorescent) samples, set the screen gain to 5 and the camera level at level 13.

NOTE: These parameters will vary based on the sample type and every sample will need to be optimized to gain focus.

3.2.12 Use standard measurement to measure the samples for 5 captures per sample where one capture-duration is for 60 seconds.

3.2.13 Save and store data after each measurement. The software will save image and video files for each measurement. The software provides output data (e.g., crystal size: 10 nM - 1000 nM and concentration) in both excel and pdf formats.

3.2.14 Calculate the average number of nanoparticles for all 5 readings for each individual sample. Analyze the data using standard deviation or standard error of the mean and use t-tests for paired analysis.

REPRESENTATIVE RESULTS:

The findings from this study show NTA can efficiently detect the mean size and concentration of calcium containing urinary nanocrystals in human urine. This was achieved by using the fluorophore, Fluo-4 AM, and nanoparticle tracking analysis. Fluo-4 AM was able to bind to both CaOx and CaP crystals. As shown in **Figure 3A**, CaOx crystals were determined to be between 50-

270 nm in size and have a mean concentration of 1.26E+09 particles/mL. CaP crystals were between 30-225 nm in size and had a mean concentration of 2.22E+09 particles/mL (**Figure 3B**). To determine if NTA could assess nanocrystals in human urine, healthy adults were asked to consume a controlled oxalate diet followed by a high oxalate load. Twenty-four hour urine samples before and after the load were collected to assess urinary nanocrystal size and concentration. Pre-oxalate urine samples contained some urinary nanocrystals ($1.65 \times 10^8 \pm 3.29 \times 10^7$ particles/mL) between 110-300 nm (**Figure 4**). In contrast, there was a significant increase ($p<0.0001$) in urinary nanocrystals present in post-oxalate samples ($7.05 \times 10^8 \pm 1.08 \times 10^8$ particles/mL; 100-320 nm) (**Figure 4**). To confirm the reproducibility of the method, samples were measured three times and there was no significant variation in technical replicates (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol for isolating and staining human urinary nanocrystals.

Figure 2: Description of Nanoparticle Tracking Analysis (NTA). (**A**) Computer and instrument set up used for these studies. (**B**) Samples are injected into an inlet tubing using a syringe pump at a continuous rate prior to filling the optical surface. Samples are then observed by the objective lens and captured by the camera as samples flow through the platform before exiting through the outlet tubing to be discarded.

Figure 3: NTA detects Fluo-4 AM labeled calcium oxalate (CaOx) and calcium phosphate (CaP) crystals. Representative graphs of (**A**) CaOx and (**B**) CaP crystals showing size distribution and concentration.

Figure 4: NTA detects Fluo-4 AM labeled 24-hour human urinary nanocrystals. Representative graph of Fluo-4 AM labeled urinary nanocrystals in 24-hour pre-oxalate and post-oxalate samples from a healthy adult on a controlled oxalate diet.

Figure 5: Technical replicates of human nanocrystals in 24-hour urine collections using NTA. Technical replicates of Fluo-4 AM labeled urinary nanocrystals in 24-hour (**A**) pre-oxalate and (**B**) post-oxalate samples from a healthy adult on a controlled oxalate diet.

DISCUSSION:

NTA has been used in the present study to assess nanocrystals in human urine using a calcium binding probe, Fluo-4 AM. There is no standard method available to detect nanocrystals in the urine. Some research groups have detected nanocrystals in the urine and relied on the use of extensive protocols or methods that are limited in their ability to quantify the samples[27,28]. This study shows a specific and sensitive method for detecting calcium containing nanocrystals in the urine of humans who participated in a dietary feeding study which consisted of ingesting a high oxalate load. The amount of oxalate consumed was equivalent to real world consumption of oxalate (e.g., ½ spinach salad).

NTA is a well characterized high resolution tool that uses Brownian motion to measure particles in solution[30]. It has been used to assess biological nanoparticles in a variety of biological

samples[31-33]. In addition, NTA can accurately predict the size as well as concentration of particles in any type of biological sample. This method does not require any labeling; however, labeling may be used to detect specific particles. Fluo-4 AM was used in this study to efficiently and specifically detect nanocrystals in urine samples. Calcium fluorescent probes were initially used to measure free cytosolic calcium[34]. Fluo-4 is an analogue of Fluo-3 whose fluorescence increases >100-fold upon binding to free calcium[35]. It was shown that Fluo-4 can also bind and label calcium containing particles[36]. In addition, Fluo-4 has been shown to assess calcium particles in the synovial fluid of patients with arthritis using flow cytometry[36]. Thus, we used Fluo-4 AM for these studies.

All samples were continuously injected into the platform for accurate detection. Determining the concentration and particle size depends on the flow rate, as a high flow rate (i.e., 50 µL/min) can affect accurate assessment of the concentration, as well as the particle size compared to a static setting and a lower flow rate (i.e. 20 µL/min)[37]. Thus, a steady slow flow rate provides accurate measurement of the number of particles present in samples. Other important parameters that might affect the particle count and size include the camera level, detection threshold, and focus[38-40]. A consistent particle measurement in samples (CV approx. 20%) was observed in the current study, which was consistent with findings from another study[39]. Lastly, the presence of nanocrystals in human urine has been confirmed using electron microscopy[29]. This research demonstrates NTA can successfully measure urinary nanocrystals from humans.

One advantage of this protocol is the use of Fluo-4 AM to evaluate calcium containing particles in solution. Another advantage is the minimal variability observed in detecting nanocrystals within samples. One limitation of NTA in this setting, is the inability to distinguish the morphology of nanocrystals. However, this method could be beneficial to detect crystalluria for predicting stone risk in individuals with a history of calcium containing kidney stones. This protocol cannot replace current methodologies but may provide new insight about urinary nanocrystals. The use of NTA to assess urinary calcium containing crystals is a novel approach that should highlight the importance of nanocrystalluria beyond standard microscopy and methods mentioned above. Additional investigations are warranted to explore the reliability of this method in the kidney stone population.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure #1

1. Urine sample
Measure urine pH and volume.

2. Centrifuge
Spin at 1200 x g for 10 minutes at room temperature (RT) and discard supernatant.

3. Clean Pellet
Wash pellet with 5 mL of 100% EtOH and Spin at 1200 x g or 10 minutes at RT (2x).

4. Prepare Pellet
Resuspend pellet with 1ml of 100% EtOH.

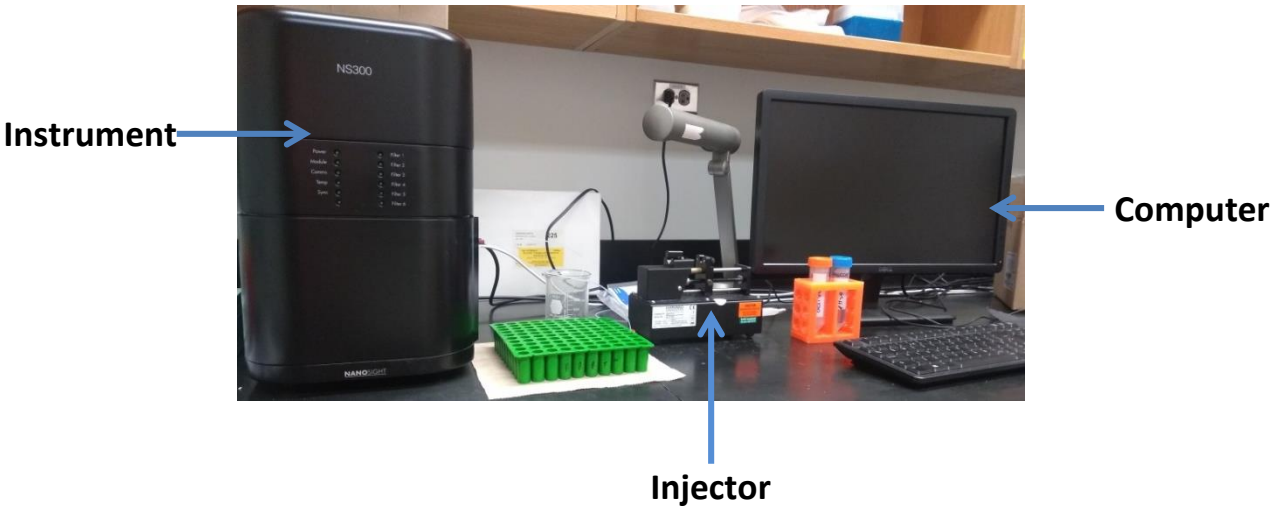
5. Stain Nanocrystals
Dilute samples in water (20x) and stain with 5 µM Fluo-4 AM for 30 mins at RT.

6. Visualize Nanocrystals using Nanoparticle Tracking Analysis (NTA)
Load and analyze samples using fluorescent filter settings.

7. Data Acquisition and Analysis
Collect and analyze data from the software.

Figure #2

A


Instrument
Computer
Injector

B

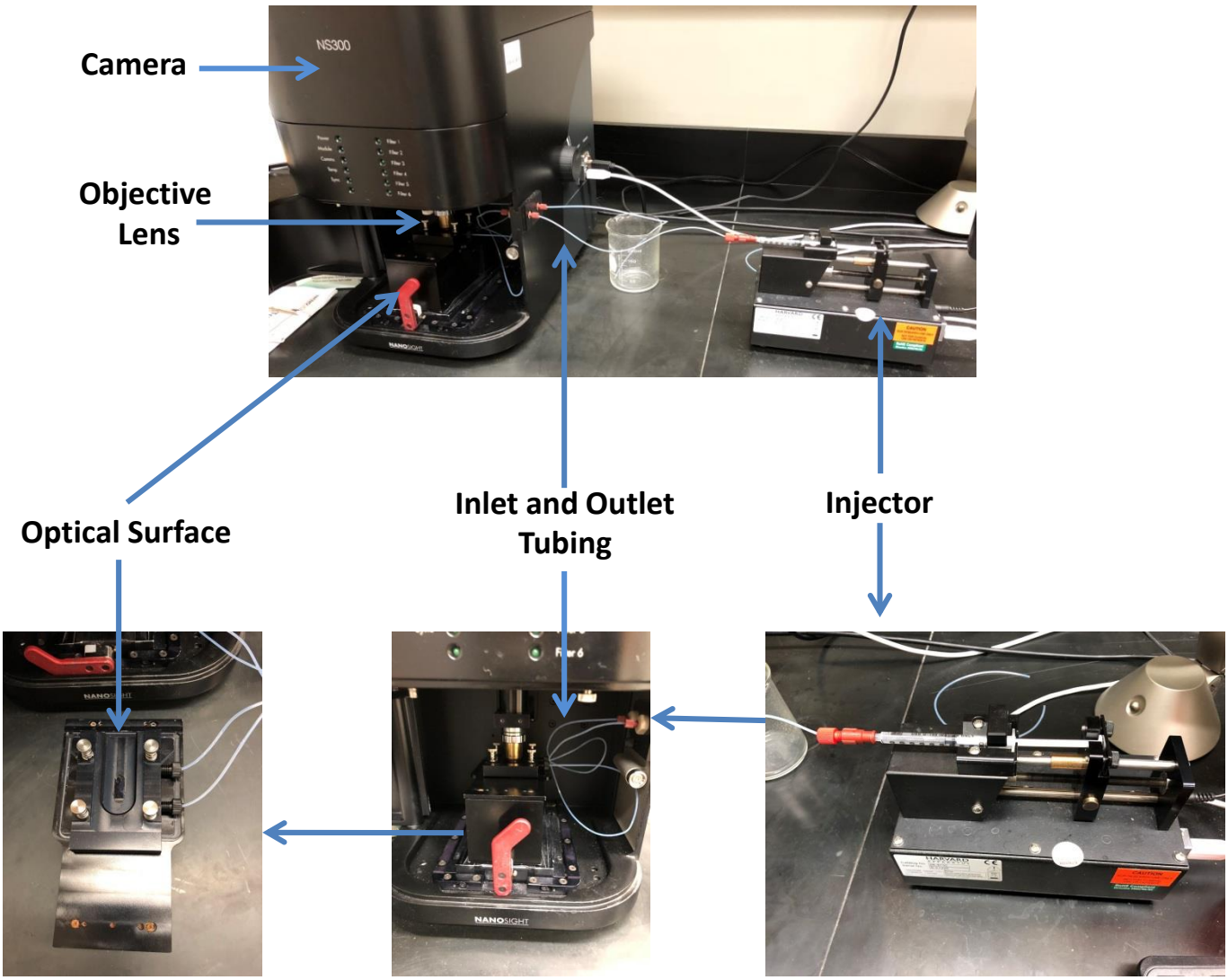
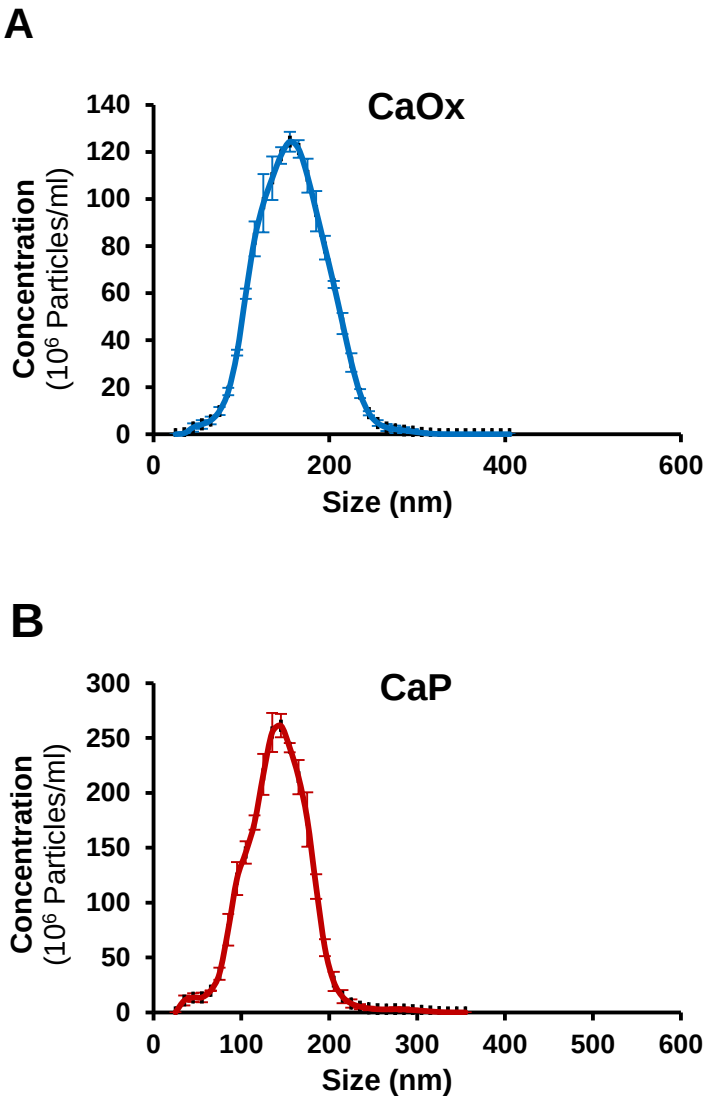

Camera
Objective Lens
Optical Surface
Inlet and Outlet Tubing
Injector

Figure #3


A
CaOx
Concentration
(10⁶ Particles/ml)
140
120
100
80
60
40
20
0
0
200
400
600
Size (nm)
B
CaP
Concentration
(10⁶ Particles/ml)
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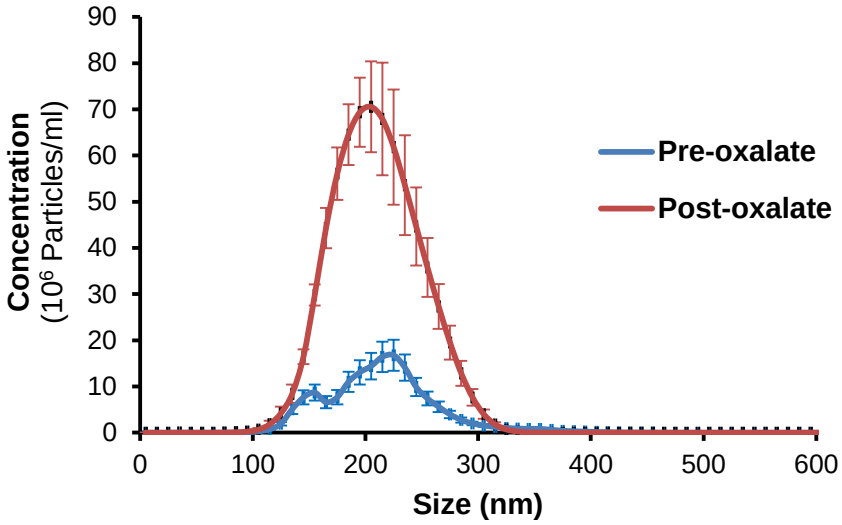
250
200
150
100
50
0
0
100
200
300
400
500
600
Size (nm)

Figure #4


Concentration
(10^6 Particles/ml)
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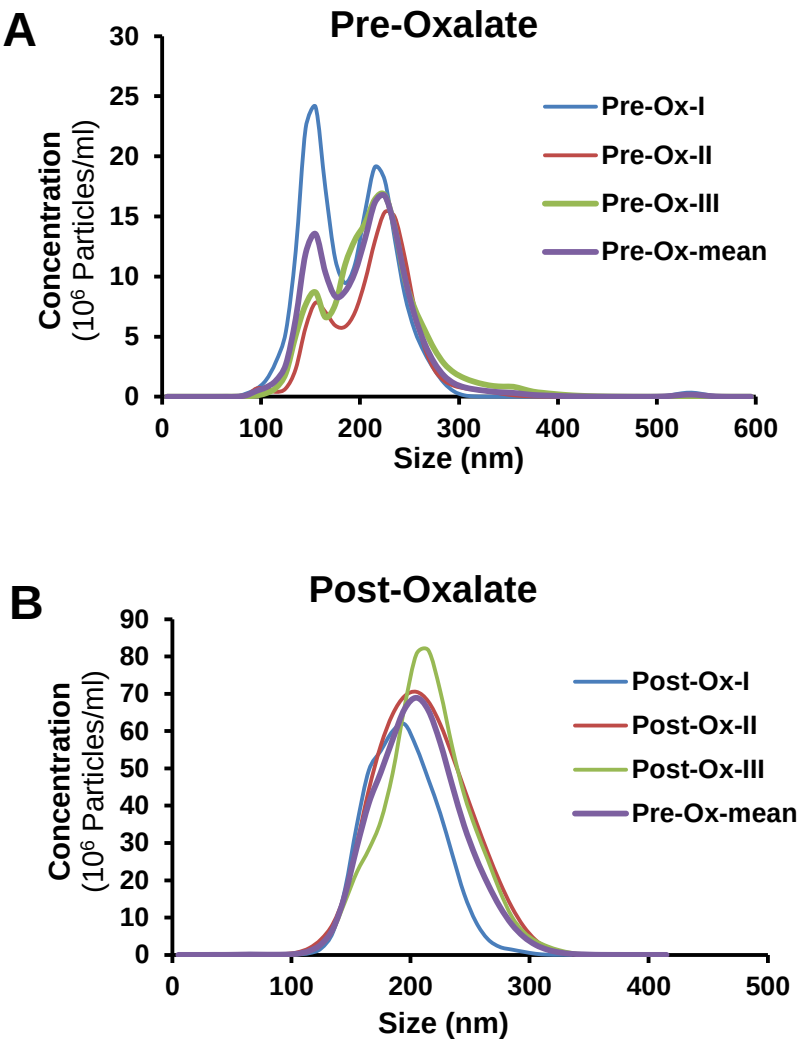
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
Size (nm)
Pre-oxalate
Post-oxalate

Figure #5


A
Pre-Oxalate
Concentration
(10⁶ Particles/ml)
30
25
20
15
10
5
0
0
100
200
300
400
500
600
Size (nm)
Pre-Ox-I
Pre-Ox-II
Pre-Ox-III
Pre-Ox-mean
B
Post-Oxalate
Concentration
(10⁶ Particles/ml)
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
Size (nm)
Post-Ox-I
Post-Ox-II
Post-Ox-III
Pre-Ox-mean

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Benchtop Centrifuge	Jouan Centrifuge	CR3-12	
Calcium Oxalate monohydrate	Synthesized in the lab as previously described[29].	—	Store at RT; Stock 10 mM
Calcium Phosphate crystals (hydroxyapatite nanopowder)	Sigma	677418	Store at RT; Stock 10 mM
Ethanol	Fischer Scientific	AC615095000	Store at RT; Stock 100%
Fluo-4 AM*	AAT Bioquest, Inc.	20550	Store at Freezer (-20°C); Stock 5 mM
Gold Nanoparticles	Sigma	742031	Store at 2-8°C
NanoSight Instrument	Malvern Instruments, UK	NS300	
Syringe pump	Harvard Apparatus	98-4730	
Virkon Disinfectant	LanXESS Energezing Company, Germany	LSP	

*Fluorescence dyes are light sensitive; stock and aliquots should be stored in the dark at -20°C.

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Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Thank you for your comments. We have thoroughly read the manuscript and corrected all errors and included abbreviations at first use.

2. Please revise the following lines to avoid overlap with previously published work: Figure 3 legend
Figure 3 figure legend has been revised.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: NanoSight; Jouan CR312 CR3-12 refrigerated benchtop centrifuge; NanoSight NS300 (text and figure); MilliQ;
All commercial language has been removed from the manuscript.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
All personal pronouns have been removed from the text.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
The protocol section has been updated.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We have added more specific steps to the protocol.

7. What were the inclusion criteria?
The inclusion criteria has been added to the manuscript.

8. Lines 95-96: With what do you mix the urine; how much of each component do you take to mix?
These details have been added to the manuscript.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
The manuscript has been formatted according to the instructions noted above.

10. Please do not abbreviate journal names in the reference list.
We have updated the journal names in the reference list.

Reviewers' comments:

Reviewer #1:

This is an exciting new technology that is very relevant for potential stratification of kidney stone formers for precision-medicine treatment. The paper is well-written. I have a few comments for the authors.

1. The authors should be commended for forging ahead into a clinical analysis area that is vastly understudied. We know that crystalluria probably provides more information on the state of urinary stone disease than do common laboratory analyses, so progress in this area is very important.
Thank you for your comments and taking time to review our work.

2. Line 53: How is it that monocytes might be exposed to urinary crystals? This seems like a non sequitur.
In the study cited, we wanted to assess the impact of oxalate (soluble and insoluble forms) on monocyte responses. Upon immune stimulation, monocytes can enter tissue and differentiate into macrophages, dendritic cells, or osteoclasts based on the cellular microenvironment. In the context of kidney stones, crystals could interact with monocytes before they differentiate into macrophages and this may induce cell death or promote them to become pro-inflammatory macrophages within the kidney. It has been reported that crystals can differentiate human monocytes into pro-inflammatory macrophages (Dominguez et al., Front Immunol. 2018 Aug 22; 9:1863.).

3. Line 67: Citing reference 24 for crystal injury to cells is probably not helpful, as that study used 'naked crystals.' It is known that the presence of urinary proteins in urinary crystals affects

the behavior of the crystals (e.g., Grover PK, et al. The effects of intracrystalline and surface-bound proteins on the attachment of calcium oxalate monohydrate crystals to renal cells in undiluted human urine. BJU International 105: 708-715, 2010).
You are correct that the reference cited used "naked crystals". We decided to cite this reference to highlight that nanocrystals might have physiological relevance in inciting injury to tissue and immune cells. We have included the reference stating urinary proteins can affect the behavior of crystals.

4. Lines 125-131: Where do these crystals come from? If it is the stock chemicals in the Table of Materials, why should there be nanocrystals in the stock chemicals? Why are oxalate and phosphate crystals done separately? What is the rationale for this distinction?
We have updated Table 1. CaOx monohydrate crystals were synthesized in the lab by adding sodium oxalate (1 mM) to $CaCl_2$ (10 mM) in HEPES/NaCl solution. We have added the reference describing this method in the protocol. CaP crystals were purchased from Sigma. Both crystals were used as a control samples. In addition, we wanted to test the ability of Fluo-4 AM to bind to calcium crystals and to compare CaOx and CaP concentrations to urinary nanocrystals.

5. Line 192: 'asses' should be 'assess'
Thank you, we have corrected this.

Reviewer #2:
In this paper, urine nanocrystals were stained with calcium-binding fluorophore Fluo-4 AM, and then calcium fluorescence labeled nanocrystals in human urine were detected by nanoparticle tracking analysis (NTA). It is concluded that oxalate loading can induce urine nanocrystals, and NTA can effectively detect nanocrystals in healthy adults. It is innovative. However, the article does not provide the photo difference between nanocrystalline and microcrystalline after dyeing, which makes readers unable to judge the feasibility of this method.
Thank you for your comments. NTA detects nanoparticles between 1 – 1000 nm in size based on the scattering of light. We have imaged microcrystals after labeling with Fluo-4 AM using confocal microscopy in our recent publication (Kumar et al., Kidney Int Rep. 2020 May 7; 5(7):1040-1051).

Reviewer #3:
Manuscript Summary:
This article demonstrates the procedure of Nanoparticle Tracking Analysis (NTA) to detect calcium fluorophore (Fluo-4 AM) labeled nanocrystals (calcium oxalate or calcium phosphate) in human urine by Nanosight NS300 system. It has been observed that after normal adults eat more oxalate foods, the amounts of nanocrystals in the urine increase significantly. But there are still the following questions:

Major Concerns:
In crystalluria, what's the correlation between nanocrystals and microcrystals in the clinic?

Thank you for your comments. There is no correlation established between nanocrystals and microcrystals in the clinic due to not having the ability to accurately quantify urinary nanocrystals. The physiological relevance of urinary nanocrystals in humans is yet to be established. However, it has been reported they may play an important role in renal damage *in vitro*.

Minor Concerns:
Are the samples obtained from 10 participants? Are some of the samples collected from the same person)? This should be clearly stated.
Yes, the samples are from 10 individual participants. We have made this clearer in the manuscript.

As has been mentioned in the section, “clinical protocol and urine collection”
The data of nanocrystal from pre-/post-oxalate diet subjects should have the standard deviation, then analyze whether there is a significant difference in the P-value or not.
We have added this information to the protocol, updated Figure 4, and added the statistical analysis results to the “Representative Results” section.