Technical Note

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Real-Time Monitoring of Urinary Encrustation Using a Quartz Crystal Microbalance

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ABSTRACT: Encrustation on the surface of urological devices such as ureteral stents leads to their blockage. However, limited tools are available for fast and real-time monitoring and modeling of the encrustation process. In this work, we have developed a model for *in vitro* study of encrustation and coupled it to an on-line monitoring QCM technique. The QCM biosensor is pre-coated with a polymer that is representative of the surface of a ureteral stent and subsequently coated with urease to facilitate crystallization of calcium and magnesium phosphate. The changes in deposition of crystals on the polymer surface are monitored quantitatively using a Quartz Crystal Microbalance (QCM) biosensor. The QCM sensor is capable of dynamic, label-free detection and has a very high sensitivity. Experimental data generated using this model shows that pre-treatment of the sensor surface with urease significantly induces early-stage encrustation as compared to untreated sensor surface, which emulates the real encrustation process. This encrustation study model has a high utility in screening studies for materials used in urological devices.

About half of the patients undergoing long-term bladder catheterization experience catheter encrustation, resulting in intraluminal blockages.[1] In 37% of catheterized patients, catheter blockage obstructs the flow of urine, which then causes urine leakage from the catheter.[2,3] Urine retention in the bladder, followed by painful bladder swelling, is another symptom of catheter blockage. In the latter case, bladder swelling can also cause urine reflux into the kidney, which can result in episodes of pyelonephritis, septicemia, and endotoxic shock.[4-6] These reasons make care of catheterized patients complicated and challenging.

Encrustation on the ureteral stent surface, eventually causing blockage, is often initiated by infection of catheters with urease producing bacteria, mainly *Proteus mirabilis*.[7-10] Soon after catheter insertion, these bacteria attach and colonize on the surface, and then form a biofilm by shielding themselves in a self-generated exopolysaccharide matrix. The urease produced by these bacteria catalyzes the hydrolysis of urinary urea to ammonia, which elevates the pH level as shown by $(NH_2)_2CO + 3H_2O \rightarrow 2NH_4^+ + 2OH^- + CO_2$. The alkaline environment enhances the precipitation of calcium phosphate and magnesium ammonium phosphate crystals present in the urine.[11-14] These crystals become trapped in the biofilm matrix and form a crystalline biofilm that can eventually block the stent.

Several *in vivo* and *in vitro* models are available to study the phenomena of encrustation in urological devices. In *in vivo* models, the test surfaces are implanted in animals such as mice, rats, rabbits, pigs,[3,15] or humans.[10,12] *In vitro* models involve placing the test surface in artificial or human urine under dynamic or static conditions. In some *in vitro* models, encrustation is induced by preconditioning the modeled urological devices or QCM biosensor surfaces with urease-producing bacteria for several days.[16,17] In both model types, sample collection is tedious and offline analysis is required to determine the amount of encrustation by using techniques such as scanning electron microscopy (SEM), energy dispersive X-ray spectrometry (EDS), UV-Vis spectroscopy, atomic absorption spectrometry, infrared spectroscopy, X-ray diffraction, or energy dispersive X-ray analysis.[1,18-22] These lead to long sample processing times, ultimately making these approaches very time consuming.

Use of quartz crystal microbalance (QCM) as an online monitoring tool for encrustation studies with *Proteus mirabilis,* has been previously reported by Gabi *et al*.[23] Koumoto *et al* have also demonstrated that hydroxyapatite crystals deposition on positively charged surfaces can be quantified using QCM.[24]

The operating principles of the QCM are described elsewhere.[25-27] Briefly, QCM measures the change in frequency associated with changes in adsorbed mass per unit area.[28] Upon mass attachment on the crystal surface, the oscillating frequency of the crystal decreases. Based on the viscoelastic properties of the deposited layer on the surface, the changes in the frequency can be back calculated to obtain the amount of adsorbed mass on the surface. The QCM sensor is capable of label-free detection with very high sensitivity, which leads to fast detection of small amounts of encrustation on the surface.

In this manuscript, we demonstrate proof-of-concept for a bacteria-free model for *in vitro* study of encrustation phenomena. In this model, we pre-coat the gold biosensor is with polyurethane (PU) polymer, commonly used as surface material for ureteral stents, to emulate protein adsorption and encrustation on the device. Since urease is known to catalyze deposition of apatite and struvite in urea, we use that knowledge to coat the sensor with urease. To demonstrate suitability of use of this model, we compare the data obtained from the biosensor against a stent device using currently existing techniques such as SEM/EDS to estimate crystal deposition. This model provides a fast, reliable, economical online monitoring system for screening of novel biomaterials for urological devices.

EXPERIMENTAL SECTION

Chemicals. Reagent grade potassium dihydrogen orthophosphate, magnesium chloride hexahydrate, calcium chloride hexahydrate, urea, calcium chloride hexahydrate, chick ovalbumin, and Jack bean urease were purchased from Sigma Aldrich.

Artificial Urine. The composition of the solutions for artificial urine was similar to the one used by Gorman, et al.[19] The composition of artificial urine and urease solutions (% w/v) were as follows:

Solution A: 0.76 potassium dihydrogen orthophosphate, 0.36 magnesium chloride hexahydrate, and 1.60 urea. Solution B: 0.53 calcium chloride hexahydrate and 0.04 chick ovalbumin. Solution C: 0.125 jack bean urease Type IX.

Solutions A and B and were prepared in DI water and stored separately to avoid precipitation of struvite ($NH_4MgPO_4 \cdot 6H_2O$) and apatite ($Ca_{10}(PO_4)_6(OH)_2$).

Materials. The material examined in this encrustation study was an aromatic polyether polyurethane (Pellethane 2363-55D, Lubrizol Lifesciences). The uncoated ureteral stents were made of Pellethane with 20 wt. % barium sulfate were supplied by Teleflex (8 Fr. experimental extrusions, not for human use), and. Sections, 3 cm in length, were cut from the body of the ureter stent. To avoid any encrustation inside the tube and to simplify calculations, the two open ends of the sections were heat sealed with hot plate at 82 °C.

QCM Technique. QCM measurements were carried out using a qCell T instrument from 3T Analytik. The sensor is comprised of a 10MHz AT-cut gold-coated quartz crystal with gold electrodes. To simulate the catheter surface, first the surface of the crystal was spin coated with Pellethane 2363-55D (10% wt/wt). The spin coating was performed at 4000 rpm for 60 s using a spin coater from Chemat Technology, Model KW-4A. The polymer-coated crystal was then heated at 60 °C in an oven for 5 hours. The PU-coated chip was then placed in the QCM instrument. The instrument is connected to a fluidic flow system, which allows running different solutions over the surface at various flow-rates. The flow rate in this experiment was 200 µL/min. The temperature of the sensor was set to 37 °C. The sensor monitors frequency changes due to crystal formation on the surface, mass precipitation on the surface, and viscosity variation between different solutions.

The experiment was started by stabilizing the frequency of the oscillating crystals by running DI water over the surface for 30 min. At that point, the running fluid was switched to solution C and the solution was flowed for 1 hour, followed by flowing DI water for 10 minutes. In the next step, salt solution, which was a 1:1 mixture of Solution A and B, was flowed over the surface for 1 additional hour to allow for encrustation. At the end, DI water was flowed again to remove all the unattached precipitate from the surface, leaving only the crystals that formed on the surface and eliminating any frequency variation caused by viscosity differences between solutions. In the other set of experiments, to evaluate the effect of urease on encrustation, the same procedure was followed except Solution C (urease) was omitted and this served as control condition

SEM/EDS and UV-Vis. Two sets of experiments were performed. In the first set, the 3 cm sections of the catheter body were immersed in urease solution (Solution C) at 37 °C overnight. Then the samples were placed in glass vials and submerged in 1:1 (Solution A: Solution B) at 37 °C for 0.5, 1, 3, 5, or 7 h, for 1 or 7 days on a shaking table at 100 rpm.

In the second set, the samples were only placed in the 1:1 (solution A: solution B) at 37 °C, without prior urease solution immersion, for 0.5, 1, 3, 5, or 7 hours for 1 or 7 days with shaking on table set at 100 rpm. At the end of each time point, the samples were washed in DI water at 37 °C for 24 hours to remove loosely attached material. The samples were then dried at 37 °C in the oven for 5 hours before the encrustation on the surface of the catheters was analyzed with UV-Vis and SEM/EDS.

To measure the concentration of calcium on the surface, the O-Cresolphthalein Chromogenic (OCPC) method was performed using a Calcium Assay Kit (K380) from BioVision. In this approach, the encrustations on the sample surface were dissolved by immersing the samples in 1N HCl at 37 °C overnight. The solution was then neutralized with 1 N NaOH. Then 10 µL of this solution was mixed with 90 µL of chromogenic reagent and 60 µL of calcium assay buffer in 96 well micro titer plates, and incubated for 10 minutes at room temperature in a dark room. A BioTeK UV-Vis Spectrophotometer was used to measure the absorbance of the solution in the wells at a wavelength of 575 nm.

The morphology of the encrustations on each sample was studied with SEM (Zeiss SUPRA 55) at the Harvard University Laboratory for Integrated Science and Engineering (LISE). Samples were first coated with 5 nm of gold using an EMS Quorum 300TD Sputter Coater, and then SEM micrographs were obtained at 100X, 1000X, and 10000X magnifications using a 5 KeV gun. The presence of calcium and magnesium ions, and their atomic percentage in the encrustations, was measured by EDS (EDAX GENESIS at 10 KeV).

RESULTS AND DISCUSSION

Encrustation Model. Unlike other *in vitro* models that mix urease or urease-producing bacteria in the artificial urine solutions to increase pH and facilitate precipitation, this experiment was designed so that urease would first adsorb on the surface. Due to the minimal amount, the pre-adsorbed urease

hardly increases the pH of the bulk solution. However, we expect the pre-adsorbed urease can still catalyze urea to ammonia locally and facilitate the encrustation reaction on the surface, which emulates the surface encrustation process of catheterization.

QCM. The change in the total frequency change and viscous damping, were measured simultaneously with the QCM. The change in mass on the sensor surface due to biomass attachment, can be calculated by incorporating these two parameters into a modified version of the Sauerbrey equation that accounts for the sensor being submerged in a viscous medium. The total frequency change is the sum of the frequency changes caused by biomass attachment Δf_b and viscous fluid on the sensor surface Δf_d, in Hz[29,30]. In this study, we used DI water before and after biomass deposition to make Δf_d a constant. Then a modified Sauerbrey equation, which relates the mass change per unit area at the QCM sensor surface to change in oscillation frequency of the crystal that is caused by biomass attachment is:

$$\Delta m_b = \frac{-C_f}{\Delta f_b} \qquad (1)$$

where, Δm_b is the change in biomass per unit area, in g cm^{-2}, and C_f is the sensitivity factor for the crystal used in the sensor (2.26×10^8. Hz cm^2 g-1 for a 10 MHz AT-cut quartz crystal at room temperature). Equation (1) indicates that an observed frequency change of 1 Hz correspondsd to a mass change of 4.4 ng cm^{-2}. C_f is a fundamental property of the QCM crystal, which is determined with the following equation:

$$C_f = \frac{2nf_o^2}{\left(\rho_q \cdot \mu_q\right)^{1/2}} \qquad (2)$$

where, n is the number of the harmonic at which the crystal is driven.

It should be stated that the Sauerbrey equation is more accurate for calculating uniform, rigid mass adsorption on rigid surface. The equation can be further modified considering the viscoelastic characteristics of both PU and urease. For PU coatings on QCM, viscoelastic parameters can be introduced into the equation, which vary with the composition of the PU.[31] For protein adsorption, the biomass can be calculated by introducing parameters from the Voigt model.[32] In this study, we simplified the calculation by neglecting these elements since the dissipative response was small compared to the frequency change during measurements.[33] The mass of our PU coating on the QCM sensor was determined to be 220 µg cm^{-2} (Δf_b = 50,000 Hz) by measuring a dry sensor without and with the coating applied.

The first set of experiments determined the effect of adhered urease on encrustation. First, DI water was flowed over the surface to reach a stable frequency, and then the salt solution mixture of solution A and B (1:1) was flowed over the surface to let the potential crystallization occur. At the end, to eliminate the effect of fluid viscosity and unbounded material in the frequency change, DI water was flowed over the surface. The mass surface density on the sensor in DI water at the beginning and the end of the experiment was equal (Figure 1a), indicating that there was no encrustation formed on the surface when urease is excluded.

In the second experiment (Figure 1b), first DI water was run over the surface until the signal stabilized, then solution C was flowed over the surface for one hour. Figure 1a shows an initial mass increase on the surface due to urease attachment of ~400 ng/cm^2. Then DI water was flowed over the surface for 10 minutes to remove the unbound urease from the surface, which caused an increase in the frequency and decrease in the mass surface density by removing some of the unbound protein. In the next step, the encrustation solution, which contained mixture of solution A and B (1:1) was run for one more hour. The mass increase on the surface is due to crystal formation, precipitation, and viscosity variation (~500 ng/cm^2). To eliminate the mass change due to precipitated crystals and study the amount of mass increase only as a result of crystal attachment to the surface, DI water was flowed over the surface again at the end to wash away all the unbound debris. The results clearly showed some (~150ng/cm^2) mass decrease in the final step. Comparing the surface mass when DI water was run at the beginning and the end step indicates an increase of 320 ng/cm^2. The results in Figure 1 indicate that adhesion of urease onto the surface accounts for 300 ng/cm^2, while encrustation adds 20 ng/cm^2 of material. The periodic fluctuations seen in Figure 1 occurring on the 100 s time scale are caused by variations in fluid flow and mechanical vibrations generated by the peristaltic pump.

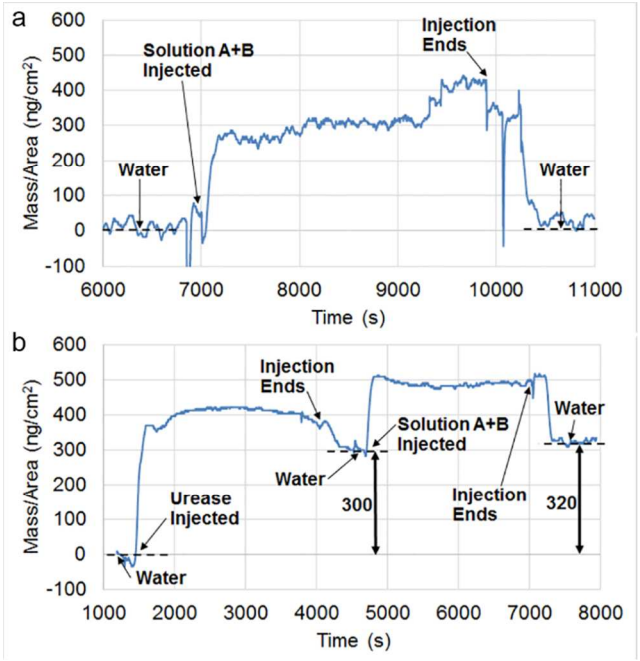

Figure 1. Graphs showing the change in mass surface density on PU coated sensor substrates over time, measured with the QCM, when a) no urease or b) urease (Solution C) was flowed over the sensor surface at the beginning of the experiment prior to exposing the surface to the Solution A+B mixture.

With a theoretical detection limit of 4.4 ng/cm^2, the urease pre-adsorbed QCM sensor can detect encrustation on surfaces in less than one hour, at a stage when nucleation initiates

without full crystal growth. In such a short period of time, no other reported assays such as SEM/EDS or OCPC can detect encrustation, either on untreated surfaces or surfaces pre-adsorbed with urease. In addition to reducing detection time, the sensitivity of this QCM model provides a useful assay to compare different surfaces and anti-encrustation agents.

In this study, pre-adsorption of urease is crucial for detecting encrustation on the surface. In a real urinary solution, urease is generated by bacteria such as *Proteus mirabilis*, which increases the solution pH by catalyzing urea to ammonia. The elevated pH precipitates the crystals from the solution and initiates encrustation on the surface[7-10]. This research further indicates that urease adsorption on a urological device can also initiate crystal nucleation under flow conditions without changing the solution pH. The amount of 300 ng/cm^2 from Figure 1b is a typically reported surface density for a monolayer of protein adsorption on an artificial surface,[34,35] which is sufficient for measurement with QCM. To confirm that pre-adsorption of urease can also facilitate long-term encrustation, both SEM/EDS and OCPC were utilized after an extended incubation time.

SEM/EDS. The SEM micrographs showed almost no encrustation formed on the surface of the samples that were not first exposed to urease solution (Figure 2a, c). The surface of these samples remained smooth after 7 days of exposure to encrustation solution. On the other hand, the surface of the samples that was first immersed in urease (Solution C) showed extensive crystal formation on the surface (Figure 2b, d). EDS was used to obtain quantitative data from the same samples by measuring the percentage of calcium and magnesium on the surfaces. The results confirm the absence of both ions on the surface of samples with no urease exposure (Figure 3), with significant increase in the amount of calcium when urease was present on the surface.

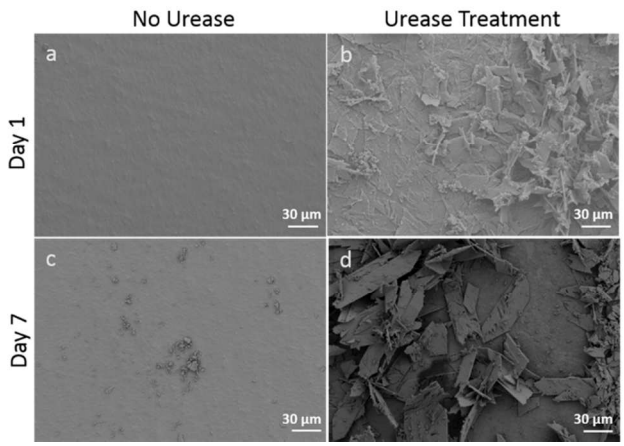

Figure 2. Scanning electron micrographs of encrustation on ureteral stentsurfaces after 1 day (top) or 7 days (bottom) of exposure to encrustation solution. Samples that were not soaked in urease solution (a, c) prior to being immersed in model encrustation solution have a much smoother surface than samples that were first exposed to urease (b, d) prior to being placed in encrustation solution.

UV-Vis. By measuring the absorbance of a calibration solution spiked with known concentrations of calcium, a linear equation to relate the absorbance and calcium concentration was obtained. The concentration of calcium released from encrustations formed on the sample surfaces was determined using the calibration curve to convert the absorbance of the OCPC solution. Figure 4 shows that almost no calcium is present on the surface when urease solution was not used at the beginning. The error bars represent the standard deviation from the mean based on three independent experiments. This result is consistent with the results obtained with EDS and QCM measurement techniques. Prior to this study, a calibration curve was generated, which provided a linear relationship between the calcium concentration in the calibration solution and the absorbance.

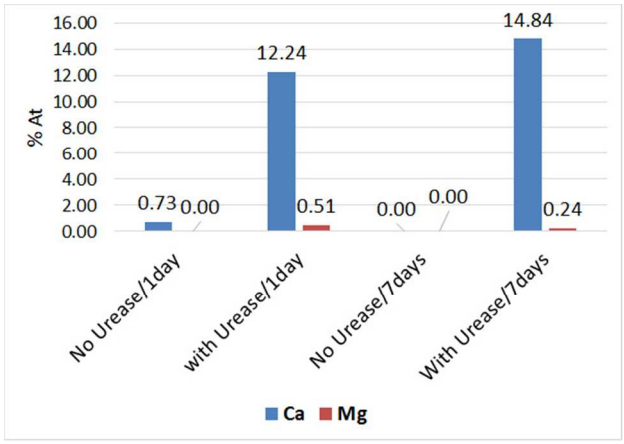

Figure 3. EDS results showing the amount of calcium and magnesium on the catheter surfaces prepared without or with urease and immersed in model encrustation solution for 1 or 7 days.

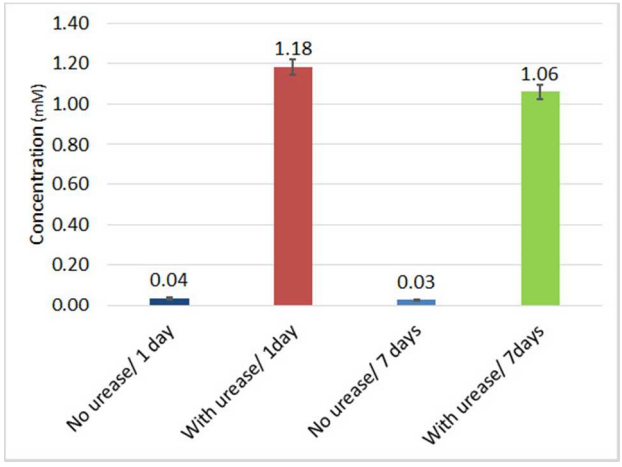

Figure 4. Measured calcium concentrations in solutions created from the encrustation on samples prepared with or without urease. The calcium in the encrustations was dissolved using an OCPC calcium assay kit and the concentration of calcium in the resulting solutions, which was measured with UV-Vis.

CONCLUSION

We have developed a new model for studying the urinary encrustation process whose readout is performed using QCM sensor. This technique provides faster results with higher sensitivity (1 ng/cm^2), which leads to a faster comparison of different substrates and chemistries for studying the prevention of encrustation. Long-term encrustation studies on ureteral stents with SEM/EDS and UV/Vis support our QCM encrustation model. Both QCM and long-term encrustation results showed urease adsorption on polyurethane surfaces played a very important role on encrustation, indicating urease-resistant surfaces may effectively reduce the encrustation on urology implants.

AUTHOR INFORMATION

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SUPPORTING INFORMATION

RawData-Figure1a-150803KOO.xls. Raw data used to generate Figure 1a.

RawData-Figure1b-150706KRV.xls. Raw data used to generate Figure 1b.

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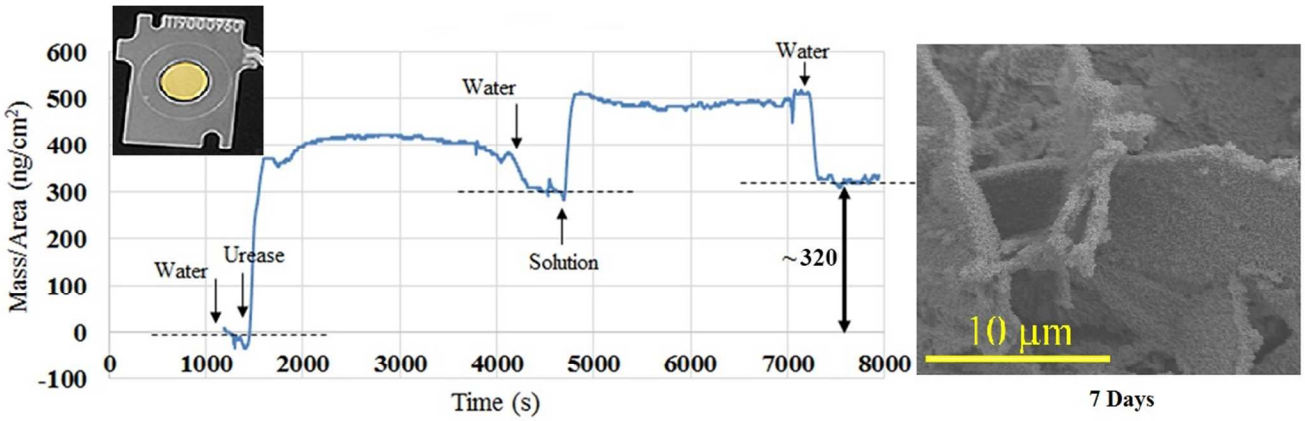
TOC


Mass/Area (ng/cm²)
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500
400
300
200
100
0
-100
0
1000
2000
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Water
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Water
~320
10 μm
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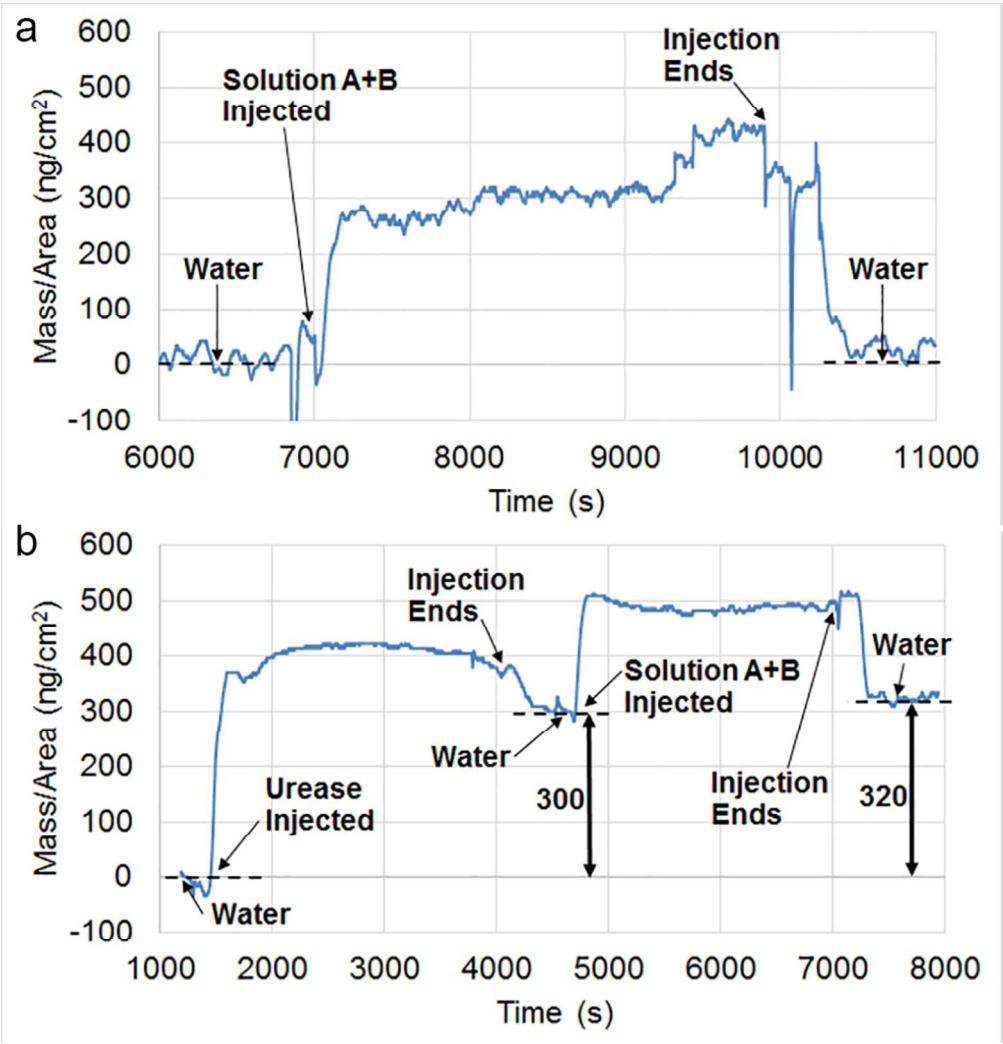

Graphs showing the change in mass surface density on PU coated sensor substrates over time, measured with the QCM, when a) no urease or b) urease (Solution C) was flowed over the sensor surface at the beginning of the experiment prior to exposing the surface to the Solution A+B mixture.

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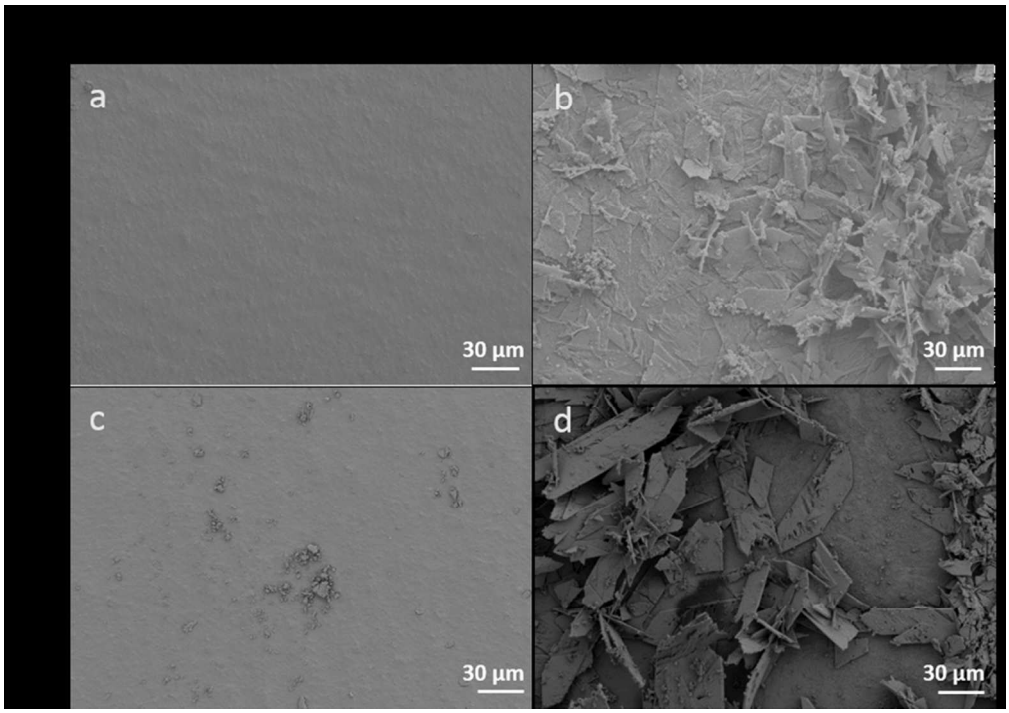

Figure 2. Scanning electron micrographs of encrustation on ureteral stent surfaces after 1 day (top) or 7 days (bot-tom) of exposure to encrustation solution. Samples that were not soaked in urease solution (a, c) prior to being immersed in model encrustation solution have a much smoother surface than samples that were first exposed to urease (b, d) prior to being placed in encrustation solution.

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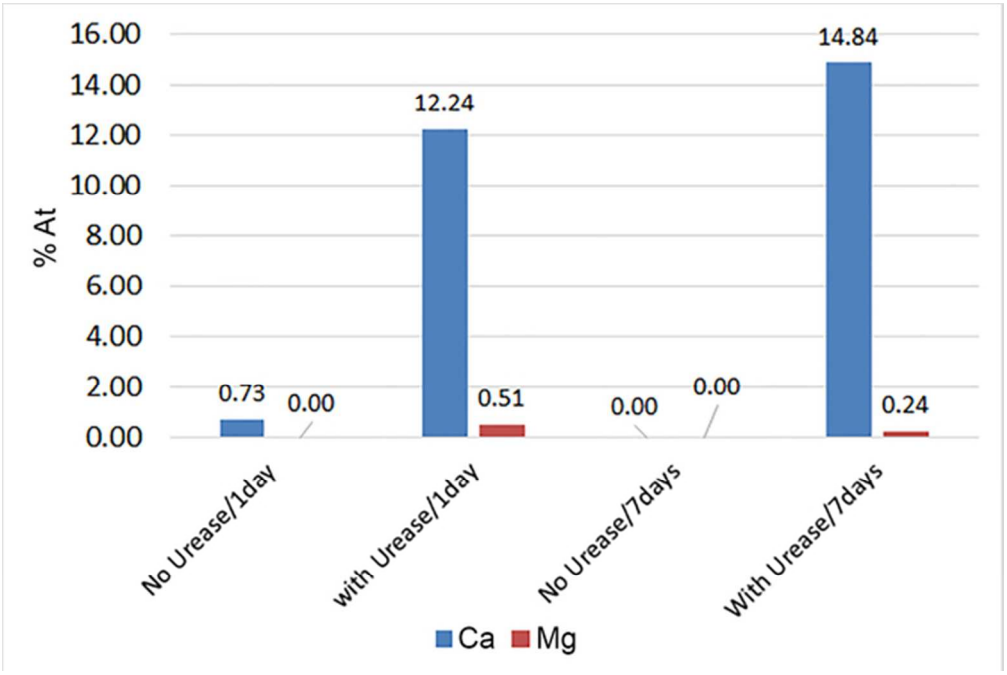


Figure 3. EDS results showing the amount of calcium and magnesium on the catheter surfaces prepared without or with urease and immersed in model encrustation solution for 1 or 7 days.

56x37mm (300 x 300 DPI)

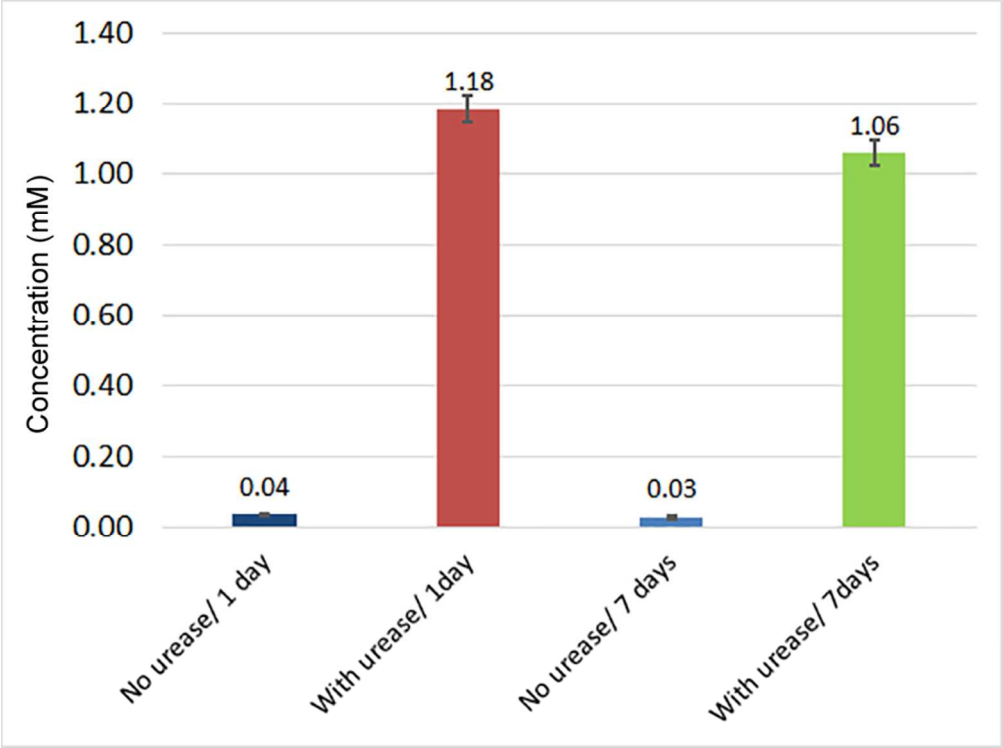
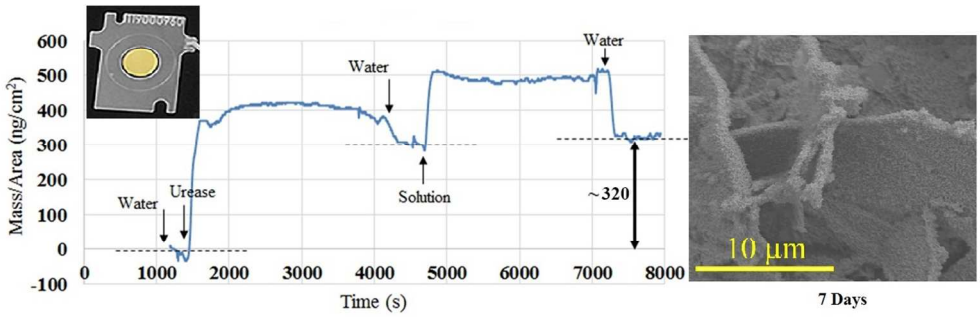


Figure 4. Measured calcium concentrations in solutions created from the encrustation on samples prepared with or without urease. The calcium in the encrustations was dissolved using an OCPC calcium assay kit and the concentration of calcium in the resulting solutions, which was measured with UV-Vis.

84x63mm (300 x 300 DPI)



117x56mm (300 x 300 DPI)