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Studies on calcium oxalate monohydrate crystallization: influence of inhibitors

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Abstract A simple model to study calcium oxalate monohydrate (COM) crystallization on different substrates is presented and the action of different potential inhibitors is evaluated and discussed. COM heterogeneous nucleation was assayed on solid surfaces as calcium phosphate, mixtures of mucin with calcium phosphate, and wax. In the presence of a non-protected non-renewed solid surface in contact with normal urine, COM crystal formation could be detected at short intervals (3 h). The most active heterogeneous nucleation capacity corresponded to calcium phosphate. In the presence of 10% mucin, owing to the renewal of the surface layer no COM crystal were detected on the pellet's surface. The study of citrate and pentosan polysulphate (a semisynthetic polysaccharide) on COM heterogeneous nucleation demonstrated some important inhibitory effects when concentration increased and time decreased. Maximum effects were selectively manifested on calcium phosphate surfaces. Only phytic acid at adequate concentration exhibited a total inhibitory capacity of COM formation, even during longer intervals (15 h).

Key words Calcium oxalate · Heterogeneous nucleation · Citrate · Pentosan polysulphate · Phytic acid

It is commonly accepted that calcium oxalate monohydrate (COM) crystal formation in renal calculi takes place through heterogeneous nucleation [4, 11]. Thus, it is clear that ordinary calcium oxalate supersaturation in urine is too low to allow calcium oxalate homogeneous nucleation and heterogeneous nucleation is a main step in COM calculus formation. Several substances have been proposed as heterogeneous nucleants. Nevertheless, in all cases it must generally be admitted that such substances must be retained in some part of the renal epithelium, normally the papilla in the case of COM calculi. Thus, calcium or ammonium magnesium phosphates are one of the most frequent substances proposed as heterogeneous nucleants of COM crystals in renal calculi. The presence of calcium phosphates have been assigned to subepithelial calcifications that, when extruded, permit COM heterogeneous nucleation (Randall's plaque) [12]. Renal infections also could generate ammonium magnesium phosphates or calcium phosphocarbonate deposits. Finally, the existence of urinary pH values above 6–6.5 can induce the formation of calcium phosphates, which may attach to the renal papilla when it is not well covered and protected by the corresponding anti-adherent glycosaminoglycan (GAG) layer [16]. Other substances that seem to exhibit significant activity as heterogeneous nucleants of COM crystals in renal calculi are uric acid and urates [3, 7]. Uric acid formation becomes important at urinary pH values lower than 5.5, at which this substance is insoluble. Other substances that can act as heterogeneous nucleants of COM crystals are mucoproteins or cellular debris [6, 13]. When these are linked to the renal papilla they can induce COM calculus formation.

To date few papers have dealt with COM heterogeneous nucleation, and even fewer with the inhibition of such processes. In this paper, a simple model for COM heterogeneous nucleation is presented, and the action of different substances, such as citrate, pentosan polysulphate (a semisynthetic polysaccharide) and phytic acid, are evaluated and discussed.

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Materials and methods

Reagents and solutions

Artificial urine supersaturated with respect to calcium oxalate ($[Ca^{2+}] = 3.5\ 10^{-3}$ M [Oxalate] $= 3.0\ 10^{-4}$ M; supersaturation $= c/c_{eq}$

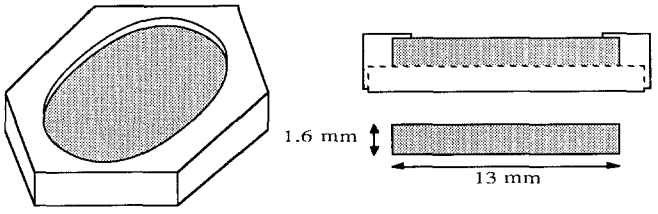

Fig. 1 Scheme of the sample holder used to immerse the pellets in the artificial urine

= 34.1) was prepared immediately before use by mixing equal volumes of solutions A and B. Solution A contained 1.93 10^{-2} M $Na_2SO_4 \cdot 10H_2O$, 5.92 10^{-3} M $MgSO_4 \cdot 7H_2O$, 8.67 10^{-2} M NH_4Cl, 0.163 M KCl and $[Ca^{2+}] = 7.0\ 10^{-3}$ M. Solution B contained 1.54 10^{-2} M $NaH_2PO_4 \cdot 2H_2O$, 1.56 10^{-2} M $Na_2HPO_4 \cdot 12H_2O$, 0.223 M NaCl and [oxalate] = 6.0 10^{-4} M. After mixing, pH was adjusted to 5.50 to avoid calcium phosphate precipitation and 0.5 ml of H_2O_2 30% was added to 1.5 l of artificial urine as disinfectant, to prevent contamination with bacteria. Chemicals of reagent-grade purity were dissolved in deionized and redistilled water.

Calcium phosphate pellets 13 mm in diameter and approximately 1.6 mm high were prepared by compressing 0.40 g of $CaHPO_4 \cdot 2H_2O$ (supplied by Panreac) with hydraulic press (Graseby Specac).

Calcium phosphate-mucin pellets of the same size as the calcium phosphate pellets were prepared by compressing 0.40 g of a mixture containing 0.36 g of $CaHPO_4 \cdot 2H_2O$ and 0.04 g of mucin (from porcine stomach supplied by Sigma) (10% of mucin) or 0.40 g of a mixture containing 0.392 g of $CaHPO_4 \cdot 2H_2O$ and 0.008 g of mucin (2% of mucin).

Wax pellets were prepared by introducing 0.2 ml of molten wax in the sample holder (Fig. 1) and left to solidify.

Measurement of calcium oxalate monohydrate heterogeneous nucleation

To evaluate the heterogeneous nucleation capacity of a given substance, a pellet of pressed or solidified substance was prepared. This was introduced in a sample holder (Fig. 1) that permitted contact of artificial urine with only a given area of one pellet face. Each pellet introduced into the sample holder was separately immersed in a controlled temperature crystallizer vessel (T = 37° C) which contained 1.5 l of artificial urine. Large volumes of artificial urine were used, in order to ensure that changes in the urine composition, due to the COM crystal growth on the active surfaces, were negligible. In absence of significant crystalluria, the heterogeneous nucleation capacity was evaluated by the number of COM crystals that appeared on the pellet surface in a given time, using scanning electron microscopy. It is obvious that the crystals seen on the substrates do not exclusively represent COM heterogeneous nucleation, since their development should imply crystal growth and aggregation. Nevertheless, it is evident that in experiments with identical conditions but varying only in the nature of the substrate, the total number of observed crystals on a substrate can be related to the heterogeneous nucleation capacity of such substrate.

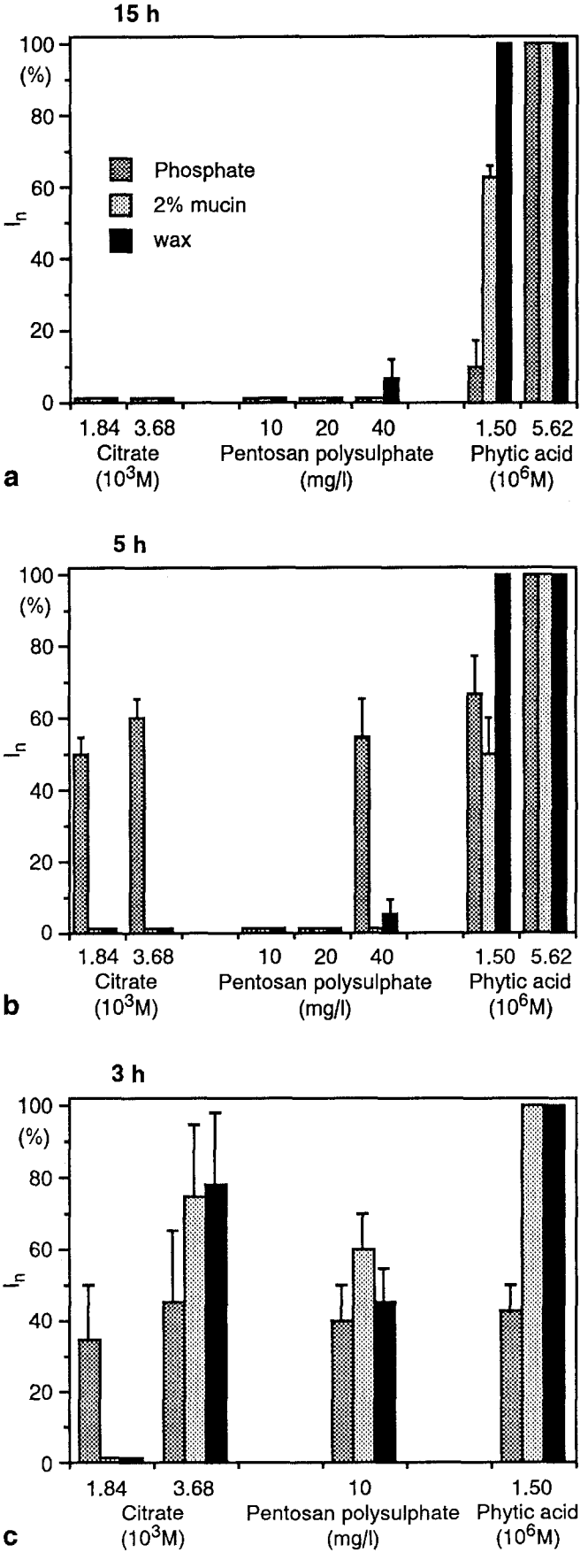

Fig. 2a–c Percentage of nucleation inhibition (I_n) exerted by citrate, pentosan polysulphate and phytic acid at different concentrations on different substrates and over different periods of time, evaluated according to the conditions stated in the experimental section. Results shown are averages of three experiments in all cases. Duration of experiments: **a** 15 h, **b** 5 h, **c** 3 h

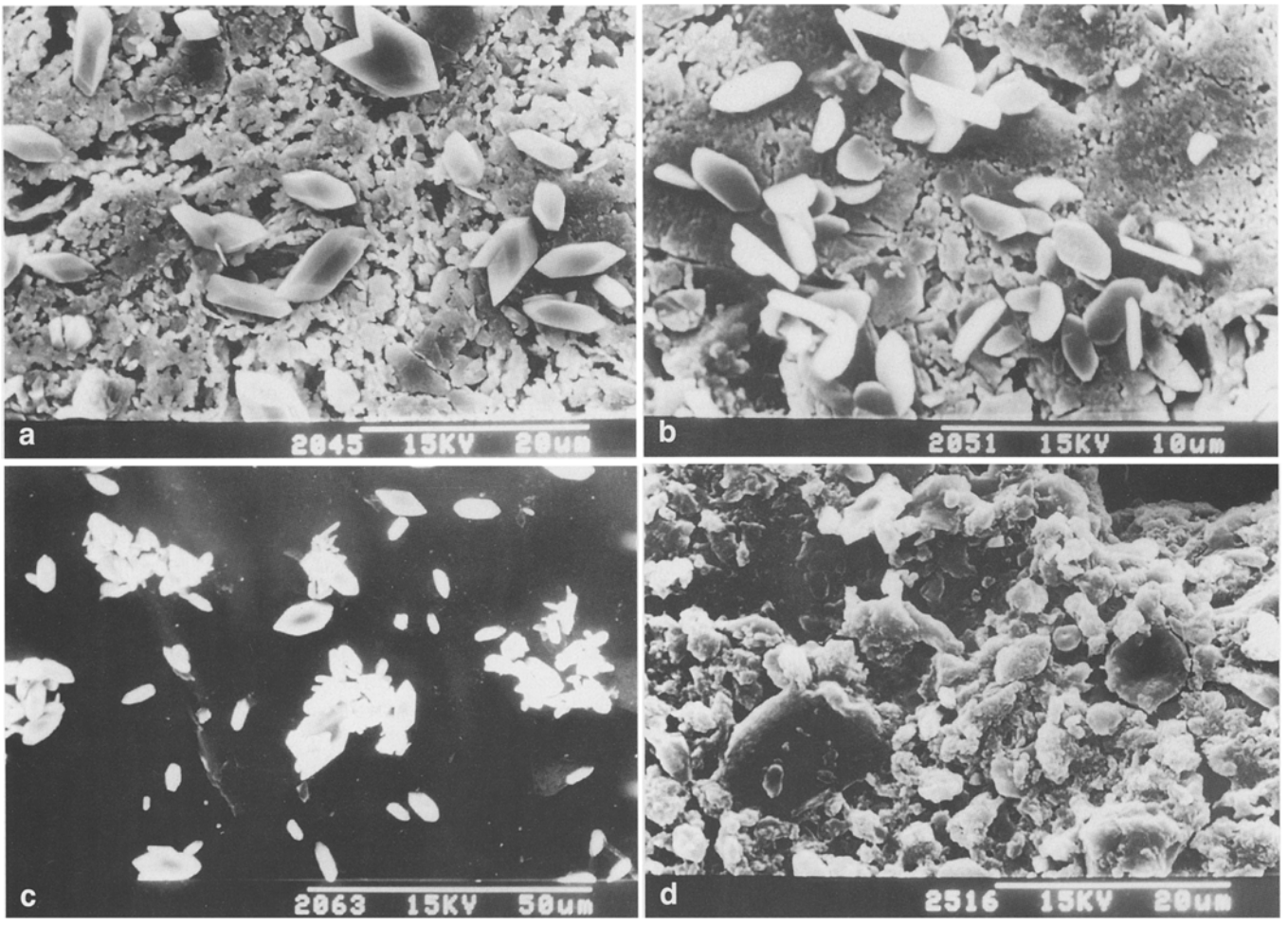

Fig. 3a–d Calcium oxalate monohydrate crystals formed on **a** calcium phosphate, **b** 2% mucin – 98% calcium phosphate, and **c** wax, at 15 h and in absence of additives. **D** Surface of 10% mucin – 90% calcium phosphate pellet in which no calcium oxalate monohydrate crystals are observed at 15 h

Effects of various compounds

The effects of citric acid (supplied by Probus), Pentosan polysulphate (supplied by Sigma) and phytic acid (supplied by Sigma) were assayed by addition of different amounts of these substances to the artificial urine. Comparison of the number of COM crystals formed on the pellet surface in the presence and absence of a particular additive permitted direct evaluation of the inhibition of COM heterogeneous nucleation. The quantity of crystals was evaluated by counting them in a 8.40 10^{-3} mm^2 area randomly selected, avoiding the pellet sides.

$$\% \text{ Nucleation inhibition } (I_n) = \frac{\text{No of crystals in absence of admixtures} - \text{No of crystals in presence of admixture}}{\text{No of crystals in absence of admixtures}} \times 100$$

To assure the reproducibility of the obtained results, each experiment was repeated three times.

Calcium-citrate complexation

Due to the high concentrations of citrate used, and considering its complexing capacity of calcium ions, in experiments where the action of citrate ions was evaluated a supplement of calcium was added in order to obtain the same calcium oxalate supersaturation value (S = 34.1) as would be found in the absence of citrate. It must be considered that a decrease in the supersaturation would imply a decrease in the nucleation rate that could not be assigned to inhibitory effects. The amount of added calcium ions was potentiometrically calculated using a selective calcium electrode (Ingold) and a potentiometer (Crison 2002). Activity of free calcium ions must be the same in citrate presence and absence, consequently when citrate was present an amount of calcium that fulfilled such a condition was added in each case. Thus, in the presence of citrate 1.84 10^{-3} M total calcium concentration was 4.0 10^{-3} M and in the presence of citrate 3.68 10^{-3} M total calcium concentration was 4.5 10^{-3} M.

Results

The results of COM heterogeneous nucleation on calcium phosphate, mixtures of mucin and calcium phosphate, and wax, in the presence and absence of different quantities of inhibitor substances and at different times, are shown in Figs. 2–4. As can be seen, in the absence of inhibitors (Fig. 3) the formation of COM crystals through hetero-

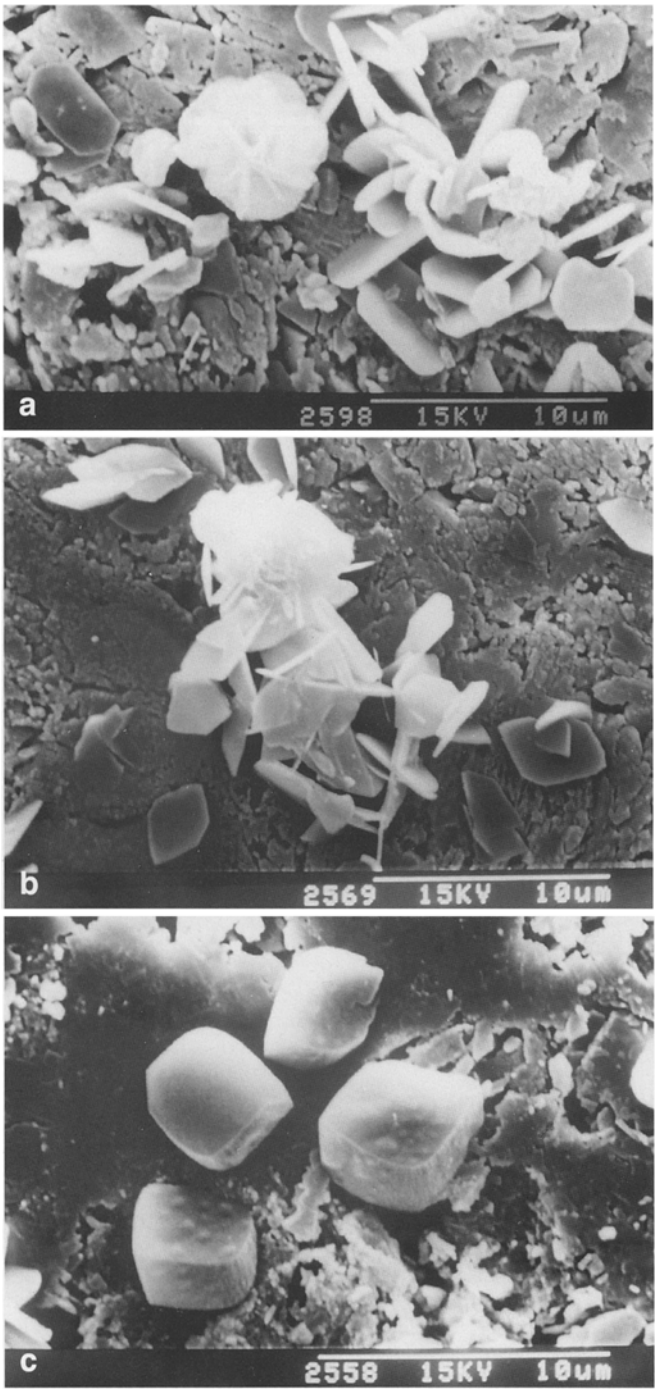

Fig. 4a–c Calcium oxalate monohydrate crystals formed on calcium phosphate in presence of **a** citrate 3.68 10^{-3} M, **b** pentosan polysulphate 40 mg/l, **c** phytic acid 1.50 10^{-6} M. In all cases, duration of experiment was 15 h

geneous nucleation on calcium phosphate, mixture of 2% mucin with calcium phosphate, and wax, was clearly detected. The number and size of the COM crystals formed depended on the duration of the experiment. The crystal morphology was clearly affected by the type of substrate used as heterogeneous nucleant in each experiment. It is very interesting to observe that in the mixture of 10% of mucin with calcium phosphate, COM crystals were not detected on the pellet (some aggregates were detected in exceptional cases).

In the presence of inhibitors, and when the experiment duration was 15 h, a total absence of COM crystals was only detected in the presence of phytic acid 5.62 10^{-6} M. It is also interesting to observe as in the presence of phytic acid 1.50 10^{-6} M, this substance acted selectively, causing total inhibition of COM formation on wax and having no effect on calcium phosphate.

When the experiment duration was 5 h, as can be seen in Fig. 2b, 5.62 10^{-6} M phytic acid caused total inhibition of COM formation, independently of the substrate; and 1.50 10^{-6} M caused total COM inhibition on wax and only partial inhibition on calcium phosphate and on the mixture 2% mucin with calcium phosphate. Pentosan polysulphate only caused partial inhibition, at maximum concentrations (40 mg/ml), on calcium phosphate. Citrate also only caused partial inhibition on calcium phosphate.

In the minimum assayed time (3 h), as can be seen in Fig. 2c, phytic acid 1.50 10^{-6} M caused total inhibition on wax and on the mixture 2% mucin with calcium phosphate, and caused partial inhibition on calcium phosphate. Pentosan polysulphate caused partial inhibition on the three assayed substrates, as did the maximum assayed citrate concentration (3.68 10^{-3} M). Citrate 1.84 10^{-3} M only caused partial inhibition on calcium phosphate.

Finally, it must be commented that in all cases, and in the presence of inhibitors, a modification of the crystalline morphology of the COM crystal formed was observed (see Fig. 4). This change of crystalline habits was observed even when no decrease in the number of formed crystals was detected.

Discussion

The role of GAGs in preventing adherence of foreign particles, seems to reach notable importance to explain the first decisive steps in COM papillary calculogenesis [5, 15]. The results of the experiments presented in this paper strongly support this postulate. Thus, in the presence of a non-protected, non-renewed solid surface in contact with normal urine, COM crystal formation could already be detected within 3 h of commencement of the experiment. The most active heterogeneous nucleation activity corresponded to calcium phosphate, as can be deduced from Fig. 3. This clearly demonstrates the COM formation risk associated with the presence of papillary calcium phosphate deposits. It is very interesting to observe that in the presence of 10% mucin, due to the renewal of the surface layer as a consequence of the transfer of mucin particles from the pellet to the solution, no COM crystals were detected on the pellet surface. Nevertheless, in spite of detecting no significant crystalluria in experiments in which 10% of mucin was present in the pellet, some typical

COM - mucoprotein aggregates were detected in the solution. This is proof of the importance of the presence of an antiadherent layer that prevents permanent deposit formation. It is also possible that the COM crystallization might be induced in solution by the mucin released from the substrate. The presence of some aggregates on the pellet surfaces, despite the absence of significant crystalluria, demonstrates the importance and significance of primary aggregation in COM papillary stone development [9]. Thus it is clear that due to the absence of crystalluria, the formation of aggregates as a consequence of crystal-crystal collisions (secondary aggregtion) could not take place. Primary aggregation represents a sort of crystal mal-growth that takes place on the surface and/or tips of already developed crystals, the so-called parent crystals [2, 10]. This process results in concretions consisting of intergrown crystals with a complex crystal arrangement.

The study of citrate on COM heterogeneous nucleation, demonstrated some important inhibitory effects when concentration increased and time decreased. It is interesting to observe that maximum effects were selectively manifested on calcium phosphate surfaces. In the presence of citrate, although a reduction of the number of COM crystals formed at longer times assayed (15 h) was not produced, the crystal size notably decreased (Fig. 4) and the shape changed. This demonstrates a strong interaction between citrate and COM-specific crystal surfaces, in agreement with observations by several authors [1, 14]. The inhibition of COM crystal growth caused by citrate can be specifically assigned to this interaction. GAGs manifested a behaviour similar to citrate, thus inhibitory effects were exhibited only when assayed times were short and preferably on calcium phosphate surfaces.

The most remarkable effects were observed in the presence of phytic acid. As can be seen in Fig. 2, no COM crystals were deteced on the studied substrates when this substance was present at $5.62\ 10^{-6}$ M, even at 15 hours; thus implying a total inhibition of COM crystal heterogeneous nucleation, on the different assayed substrates. It is very interesting to observe how, when phytic acid was present at $1.50\ 10^{-6}$ M and the experiment duration was 15 hours, a selective inhibition of COM heterogeneous nucleation was observed in function of the assayed type of solid surface. The presence of phytic acid also affected the size and shape of the formed COM crystals, as must be expected due to the strong interaction with the COM crystal growth [8].

In conclusion, adequate urothelium protection and renovation seems to play an important role in preventing COM urolith development in such a way that some inhibitor substances of COM heterogeneous nucleation, such as citrate and pentosan polysulphate, can exert a significant but not decisive action. Only phytic acid at adequate concentration exhibited a total inhibitory capacity of COM formation.

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