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ARTICLE

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In vitro calcium oxalate crystallisation methods

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Abstract In vitro calcium oxalate crystallisation has been, and will continue to be, of fundamental importance to urolithiasis research. Many different methods have been employed which differ qualitatively and quantitatively in the extent that they reproduce aspects of the renal system or in their ability to distinguish different aspects of crystallisation activity. Whatever system is used there are three key aspects that are worth bearing in mind. Firstly, a major controlling factor will be the prevailing supersaturation and other physicochemical considerations, secondly, during the course of the reaction different processes may come into play and thirdly, the processes we are trying to model take place in a dynamic biological environment. Different approaches to the study of crystallisation can be classified in many ways, such as the process or analytical technique but at a more fundamental level it is helpful to focus on the changes in supersaturation during the course of the reaction. A steady state supersaturation is more likely to be representative of the intra-renal situation than a system which decays to the equilibrium position. The constant composition method and the mixed suspension mixed product removal method both achieve a steady supersaturation.

Keywords Calcium oxalate · Crystallisation · Continuous crystalliser · Constant composition

Introduction

Crystallisation methods used in urolithiasis research come in many guises; in vivo, in vitro or in the presence of cultured cells and they may be fully objective, semi quantitative or subjective; they may be involve the use of complex expensive equipment or be simple and cheap. The motivations for different experiments can also be as many and varied as the methods used. The key to a worthwhile crystallisation is to match the objective with the appropriate procedure. In this review I will concentrate on quantitative, in vitro methods for studying calcium oxalate (CaOx) crystallisation processes. Within these limits there are still many different options available to choose from and these vary in the degree of ''naturalness'' from simple experiments in defined inorganic solutions to whole urine experiments which replicate some features of urine flow dynamics. Irrespective of how much verisimilitude is achieved it is incumbent on authors not to extrapolate their findings beyond their limits or go on to draw unwarranted conclusions for physiological significance [1]. The intention of this review is to give a general description of methods available and readers should consult some earlier reviews [2, 3] and the original papers for practical details and information on the mathematical processing of the data.

Crystallisation is a physical chemical process involving a change of state from solution to solid. The supersaturation, which is a measure of the chemical energy available for this, is a crucial factor and governs all aspects of crystallisation such as nucleation, growth and aggregation. As the reaction proceeds, the supersaturation will decline (unless replenished) and this in turn will impact upon the kinetic behaviour of the crystallisation processes. While the physical chemistry and kinetics are always important, the process of stone formation takes place in biological environment. It can be helpful to keep in mind the relative roles of supersaturation, kinetics and biology when designing experiments, and the setting of the selected model can be thought as being positioned at some point in a space defined by these three dimensions (Fig. 1).

Classification of methods

When considering the different approaches to CaOx crystallisation, they naturally fall into different

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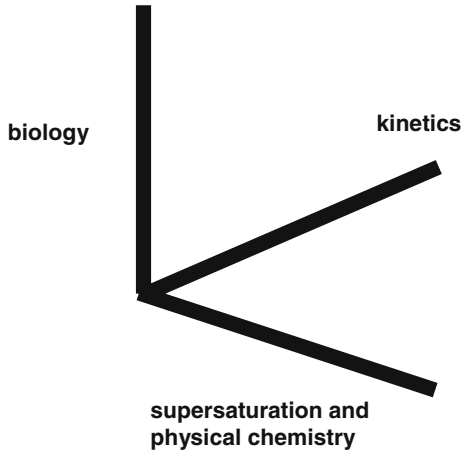

Fig. 1 Crystallisation and stone formation can be thought of as a problem in three dimensions and different methods vary in where they are positioned in this space

categories which can be conveniently be thought of as belonging to differences in the process used, in the analytical techniques applied or in the profile of supersaturation changes during the course of the crystallisation (Fig. 2).

Most of the CaOx crystallisation methods used in urolithiasis research have been adapted from techniques widely applied in other fields such as materials science or chemical engineering. Of the different categories suggested in Fig. 2 the supersaturation profile (Fig. 3) is arguably the most useful. Not only is supersaturation the fundamental driving force for the reaction but also the way in which supersaturation is brought about in the kidney is quite different from most methods used in other crystallisation procedures. Plasma is filtered in the glomerulus. As the filtrate passes through the renal tubules to the tips of the collecting ducts at the papillae it is modified by reabsorption or secretion and water removal. This brings about changes in the saturation as the developing urine passes through the system. Because it is a continuous flow system, the composition at any one point within a tubule remains constant as long as external factors (e.g. renal blood flow, degree of hydration) remain stable. These three features of urine production, continuous flow, supersaturation developed by water removal and relatively constant supersaturations at different points are each reproduced in different methods but no one method models mimics all three at once.

Supersaturation decay

Simple batch (discontinuous) systems are probably the most widely used methods in crystallisation research. Because soluble salts of calcium and oxalate are available it is a simple matter to bring together two solutions which when combined produce a supersaturated solution of CaOx.

If the supersaturation exceeds the formation product (FP) (or metastable limit) then crystallisation proceeds

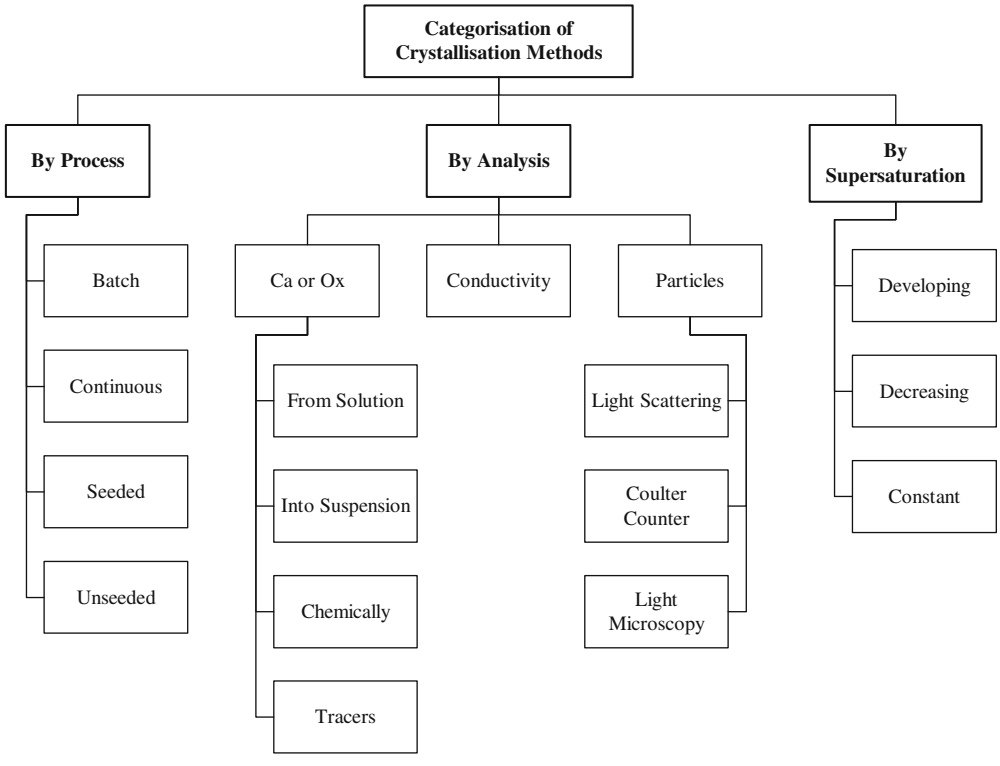

Fig. 2 Crystallisation methods can be categorised by the type of procedure, by the analytical technique or by the supersaturation profile

spontaneously (curve A in Fig. 3). Because of the difficulty in preparing particulate free solutions in vessels with no surface defects (both at the sub-micrometre scale), this crystallisation will almost always be dependent upon heterogeneous nucleation. Once crystallisation has started, the supersaturation will reduce as calcium and oxalate are removed from the solution, eventually reaching an equilibrium position with a supersaturation ratio of 1. As the supersaturation decays, crystals may be nucleating, growing and aggregating. If one measures the amount of calcium or oxalate removed from solution or incorporated into precipitate at a fixed time (or times) then one can estimate the growth (crystal nuclei only occupy a very small proportion of micrometer diameter crystals). Nevertheless, it is important to remember that this growth is not independent of the nucleation and aggregation as a rapidly nucleating suspension will allow more crystals to grow and aggregation will impact upon the crystal surface area presented to the solution. If particle size distributions are measured during this type of procedure then it is difficult to disentangle the effect of different growth processes on crystal numbers or crystal volumes. Because of the difficulties in deciphering the role of nucleation, growth or aggregation, most of these experiments are performed as a comparison between a test condition and a control, rather than quantitating some parameter directly.

An advantage of a spontaneously precipitating batch system is the ease with which it can be applied usefully to whole urine, simply by adding a small volume of a concentrated calcium, or more commonly an oxalate, solution. In particular, this can enable an estimate of the FP to be made. Usually this is done by testing increasing concentrations of added oxalate in order to identify the least challenging conditions required to initiate crystallisation. It is important to recognise that the FP is not well defined in thermodynamic terms and any empirical measure will necessarily be dependent on the method used to detect the onset of crystallisation. Because nucleation in whole urine will almost certainly be heterogeneous, an estimate of FP can be taken as an indicator of the heterogeneous nucleating capacity of the sample. Many methods have been described with this in mind [2–8]. Turbidity or light scattering is a simple method for estimating when crystallisation has occurred and there are some methods available to extract quantitative data on growth and nucleation by following the kinetic course of the turbidity profile [4–6, 9].

Although very different protocols are used in these methods, no one procedure can be said to be inherently superior to another. The choice might well come down to the particular experimental requirements or the equipment available. Such systems can be used to make intra-procedural comparisons but inter-procedural comparisons would not be valid. For example, we have recently compared the crystals produced by six unseeded batch methods which differed only in the details of the addition procedure and the quantity of calcium and oxalate added to fresh whole urine. Using the same pooled urine, with all experiments performed at the same time, we found enormous variability in the size, surface area, degree of aggregation, dominant hydrate and crystal morphology of the product [10].

Seeded batch systems (curve B in Fig. 3) are performed by setting up a metastable supersaturated solution and introducing seed crystals. These then grow and possibly aggregate. As nucleation is not taking place in this system interpretation of quantitative results is inherently simpler and it is possibly using particle size distributions to distinguish between aggregation and growth effects [11–14]. Because of the difficulty of setting up a metastable solution of controlled composition, seeded methods have generally been applied in well defined media rather than whole or nearly whole urine. As with unseeded systems, there is enormous variability in the details of the procedures employed. In 27 papers 6 pH values, 7 oxalate concentrations, 9 detection methods, 10 calcium concentrations and 13 seed densities were used [2].

A particular format of unseeded crystallisation which focuses on the induction of nucleation is with the use of Langmuir monolayers. In this a monomolecular film is formed at the air–water interface, usually composed of amphiphilic molecules. As an ideal two-dimensional system it acts as a model of biomembranes. With a metastable supersaturated solution of CaOx in the water layer, nucleation can be induced at Langmuir layers of phospholipids. This allows the influence of the nature and structure of the monolayer to be investigated along with the effect of including various potential crystallisation modifiers [15–21].

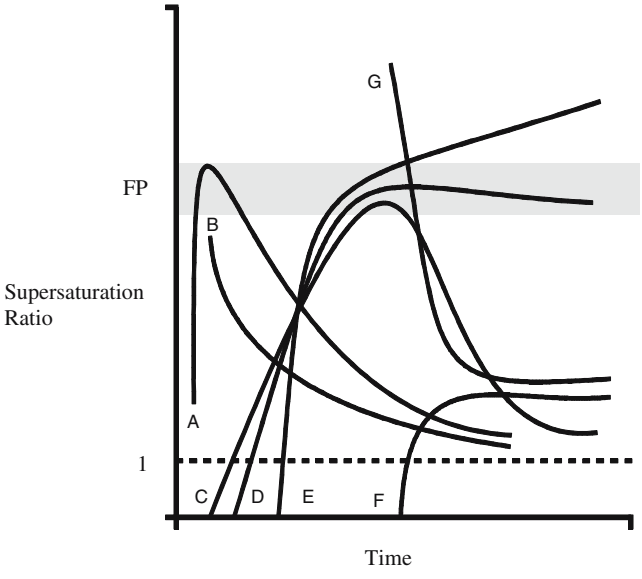

Fig. 3 Supersaturation profiles during different crystallisation procedures

Supersaturation developed slowly

There have been relatively few procedures which adopt this approach in urolithiasis research and they seem to have fallen out of favour in recent years. The common feature is a relatively slow method for increasing the concentration of calcium and/or oxalate which is maintained after crystallisation has initiated. The precise supersaturation profile will reflect the relative rates of the two processes (curves C, D and E in Fig. 3). Methods which use reverse osmosis [22–24] or evaporation [25–30] were specifically designed to mimic the water removal generation of urinary supersaturation but it is not obvious that any direct insights were achieved through this approach. Perhaps more rewarding in this respect has been the use of other methods with media designed to mimic the concentrations in different regions of the renal tubules [31–33]. Other means of slowly developing the supersaturation often rely on diffusion of calcium and/or oxalate solutions through gels or of vapour diffusion of diethyl oxalate, which is subsequently hydrolysed to release oxalate. The relatively slow growth that can occur under these conditions can give rise to large crystals suitable for particular structural/morphological examination but these approaches do not seem to have received much attention since last reviewed [2, 3]. By their nature, these diffusion techniques allow relatively little control of the supersaturation at the point of crystallisation. This has been overcome in a technique applied to calcium phosphate crystallisation [34], where the concentration of diffusing reactants was held constant by the same method as utilised in the constant composition procedure (described below).

Constant supersaturation

The urinary system is in a state of continuous flow with the effect that the contents of each region are being continually replenished. This means that the environment at any point is relatively stable and that, even if crystallisation is taking place, the supersaturation will not be changing as a result. This behaviour can be reproduced in two methods that have been quite widely applied in urolithiasis research; the constant composition method and the mixed suspension mixed product removal method (MSMPR).

As first described, the constant composition method used a metastable solution seeded with the appropriate crystals [35, 36]. As crystal growth starts, the ionised calcium and oxalate begin to decrease in equimolar proportions. The fall in ionised calcium is sensed by a calcium electrode and the output from this controls a syringe pump which is driven to restore the original conditions (curve F in Fig. 3 and Fig. 4). The rate of pumping of new reactants is therefore directly equivalent to the crystal growth rate. This method can be used to study the reaction kinetics [36, 37], to quantify inhibitor activity [38] or to estimate interfacial tensions [39]. The method can also be used in an unseeded mode to examine nucleation [33, 40, 41]. Because metastable solutions are inherently unstable, they will eventually nucleate and begin to crystallise. The lag time for this to take place is an indication of the nucleating efficiency of the medium.

The MSMPR method was developed by the Chemical Engineering community and taken up for urolithiasis research because of the realisation by Finlayson [42] of

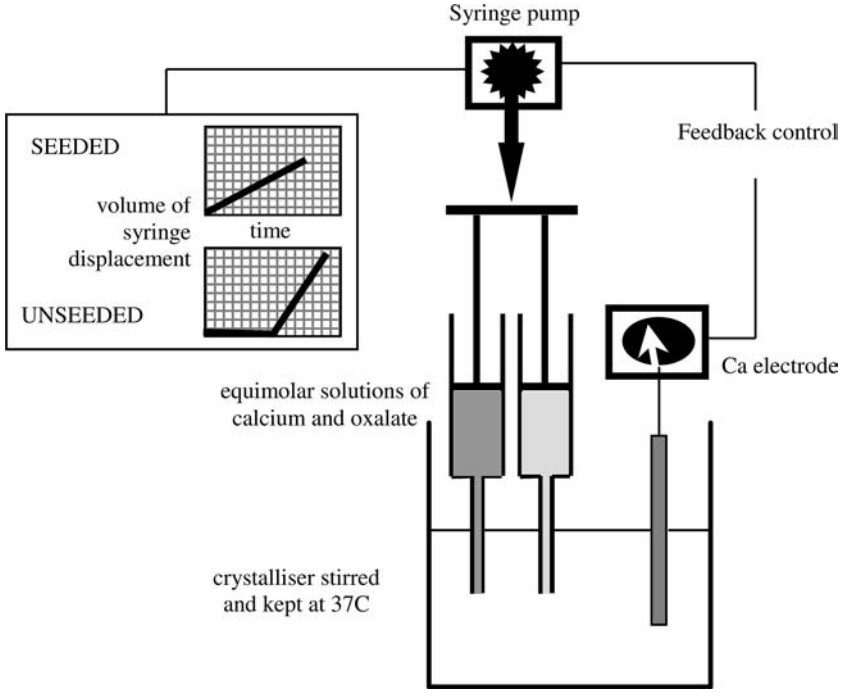

Fig. 4 Constant composition method. When crystals form the drop in ionised calcium is sensed by the electrode which controls a syringe pump to deliver calcium and oxalate solutions so as to restore the balance. Can be performed as a seeded experiment to measure growth rates or as an unseeded system to measure induction times, as an indicator of nucleating capacity

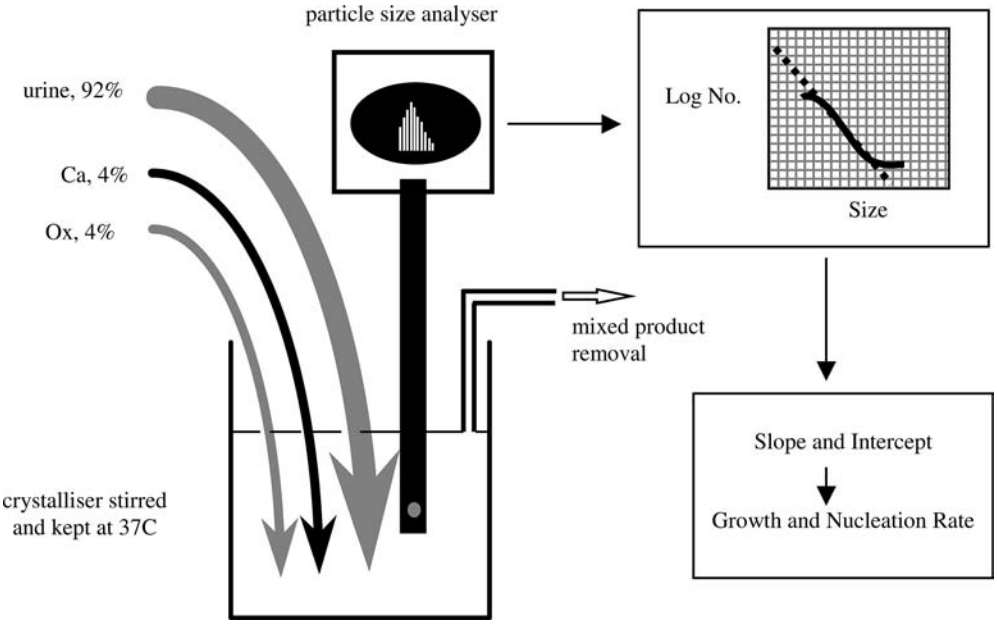

Fig. 5 The mixed suspension, mixed product removal (MSMPR) method for use with nearly whole urine. The total input flow (Q) is proportioned as 92% urine and 4% for each of a calcium and an oxalate solution. The output flow maintains a constant volume (V). After 6 to 8 residence times ($=Q/V$) the system reaches equilibrium and the particle size distribution is measured, from which the growth and nucleation rates are calculated

the analogy between a pair of MSMPR crystallisers in series and the collecting ducts and renal pelvis. The method relies on a continuous input of reactants and removal of the mixed suspension. The system has a characteristic residence time (volume/flow rate) and after about 6–8 residence times it comes to a dynamic equilibrium with a stable supersaturation and particle size distribution (curve G in Fig. 3). From the particle size distributions, it is possible to calculate both the nucleation and growth rates [2] and estimates of the aggregation behaviour can also be obtained [43]. Early experiments [44–47] were limited by the volume of the crystallisers and only dilute urine could be used. By reducing the chamber volume and reconfiguring the flow system [48, 49] we were able to use the system with nearly whole urine (Fig. 5) [50, 51]. Suziki et al. [52] used a similar system to investigate deposition of crystals onto fragments of renal stone material and we have developed this into a method for growing clinically significant sized stones [53] and operated this on a larger scale as a stone farm [54, 55].

Conclusion

There are many different methods available for experimental CaOx crystallisation research. To differing degrees, each has different strengths and weaknesses and while the constant composition and MSMPR methods scored well in a questionnaire at a workshop on the subject [1], no one method could be seen as clearly superior to all others. The focus here has been on different classes of procedures yet even within seemingly very similar methods great variations in the detailed protocols can apply with significant consequences for the outcome [10]. It is clear that comparison of results obtained by different methods should only be made cautiously and if there is a requirement to make a quantitative comparison then the same procedure should be used [56].

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