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ACKNOWLEDGEMENTS. We thank the Director, Central Agricultural Research Institute, Port Blair for facilities provided. This work was carried out under the aegis of a R&D project sponsored by Department of Biotechnology, New Delhi.

Received 28 March 2005; revised accepted 7 February 2007

Modification of gel technique for micro-crystals of biomaterials: *In situ* growth and dissolution studies

Bharat B. Parekh[1], M. J. Joshi[2,*] and A. D. B. Vaidya[3]

[1]Institute of Diploma Studies, Nirma University of Science and Technology, Ahmedabad 384 481, India
[2]Crystal Growth Laboratory, Department of Physics, Saurashtra University, Rajkot 360 005, India
[3]Bhavan's SPARC, Juhu Scheme, Mumbai 400 049, India

In the process of biocrystallization and biomineralization, the growth of crystals occurs under tissue conditions prevalent in the body. Many biomaterial crystals are initiated at micrometre size and have different morphologies. Such micro-crystals play a pathogenetic role in different diseases. Hence, an *in vitro* method to induce micro-crystals of biomaterials is desirable as a model to study the morphogenesis as well as its reversal by intervention. A modified gel technique for the growth of micro-crystals of biomaterials is proposed. The growth of urinary type-calcium hydrogen phosphate dihydrate (CHPD) is carried out to verify the utility of the technique. Growth inhibition and dissolution of CHPD crystals by solutions of different concentrations of citric acid is studied *in situ*. This technique is economical, needs less amount of material and can be used in batches in aseptic conditions with *in situ* observations under optical microscope. For *in vitro* studies of growth inhibition and dissolution of biomaterial crystals by different solutions and herbal ayurvedic plant under aseptic conditions, the present model provides a screening tool for drug development of urinary litholytic agents.

Keywords: Biocrystals, gel growth, litholytic agent, micro-crystals, urolithiasis.

BIOCRYSTALLIZATION and biomineralization phenomena are the root cause of several diseases in humans; the common examples are crystal-induced arthropathies, urinary calculus, calcified plaque in the arteries, gall bladder stone, cataract, etc. Gout is a metabolic disorder of uric acid leading to arthritis; other crystals also induce arthropathy (joint disease). Deposition in the synovial cavity of several crystals, such as hydroxyapatite, monosodium urate monohydrate and calcium pyrophosphate dihydrate leads to arthropathy[1–5]. The World Health Organization has recognized the problem of arthritis and declared the period 2000–10 as the bone and joint decade[6]. Similarly, urinary stone formation leading to renal colic is an old and widespread problem. In urinary calculus several min-

*For correspondence. (e-mail: mshilp24@rediffmail.com)

eral crystalline components are found; calcium oxalate monohydrate, calcium oxalate dihydrate, calcium hydrogen phosphate dihydrate (CHPD) and ammonium magnesium phosphate hexahydrate are the most common ones[7]. According to an estimate, nearly 12% of the population of the European Union suffers at least one episode related to urolithiasis[8]. In the Saurashtra region, Gujarat, there is high incidence of urinary lithiasis[9].

The process of biocrystallization of cholesterol plays a significant role in arthrosclerosis leading to cardiovascular disease, viz. coronary events and strokes. Gall stone disease can also occur due to cholesterol crystals besides bile-salt crystals. In 1986, coronary artery bypass grafting accounted for less than 10% of all cardiac surgeries in India; however, it is expected to reach almost 60% now[10]. Also, the gall stone disease afflicts nearly 12% of the population in USA[11,12]. Cataract is also common among the aged. It has been reported that the face-centred cubic (FCC)-type ferritin crystals in cataract play an important role in light scattering[13,14].

It is important to notice that in most cases the growth of biocrystals occurs either in the soft tissue, blood vessel or cavities. The growth of crystals is slow and initiate at micro-meter size. It is important to understand the pattern of growth and morphology of the micro-crystals. Therefore, we have proposed a model system based on the gel growth of micro-crystals, which can facilitate *in situ* observations of crystals in the process of their formation and growth. This model would serve as a good screening *in vitro* method for studying the inhibition or dissolution of crystals at early stages. It would help evolve preventive modalities for those prone to stone formation.

Growth of various crystals by the gel method is well explored[15,16]. However, the growth of micro-crystals in the gel environment has not been well explored, except for van Ivan[17]. The growth of crystals in a gel medium is a result of slow diffusion of two reactants and formation of nuclei due to the reaction between them and the subsequent supply of nutrients resulting into further growth of crystals by accretion. This has been explored in detail in earlier studies for the growth of urinary crystals and their inhibition or dissolution[18–22].

Glass slides with cover slips and petri dishes were used as micro-crystal growth apparatus. The glass slides were arranged in the petri dish in the form of a plus sign, where the lower slide was used for support and the upper slide used for micro-crystal growth. To grow micro-crystals in a silica-gel medium, a sodium meta-silicate solution of appropriate specific gravity was mixed with a suitable molar concentration of a weak acid so that the desired pH can be set for the mixture. Thereafter, with the help of a suitable glass dropper a small drop of this mixture was put in the middle of the glass slide. A cover slip was then placed on this drop in such a manner that it floated on the mixture and covered almost the area of the cover slip, without any spillage beyond the cover slip. To assure that the gelling process occurred properly without drying up of the solution, the slides were placed in the petri dish in such a way that water poured in the petri dish did not touch the cover slip, but remained slightly below the upper surface of the slide. After setting the gel, water was sucked away from the petri dish with a dropper. Thereafter, suitable concentration of the ionic salt solution was poured gradually using the glass dropper so that it covered the slide up to the level of the cover slip. The poured solution diffused through the gel and reacted with the impregnated weak acid in the gel medium, which resulted into the growth of micro-crystals within 24 h. If the cover slip had a diameter of 18 mm with the total area of 254.34 sq. mm, and the drop put on the slide to form the gel was of the order of 10 µl, then the thickness of the gel could be estimated to be of the order of 0.039 mm. Figure 1 shows the schematic diagram of the glass slide arrangement in the petri dish.

The slides were observed regularly using an optical microscope and photographs taken using an attached digital CCD colour camera or a time lapse camera.

The slides were put in batches for the growth inhibition and dissolution studies in aseptic conditions. Different inhibitory solutions and extracts of ayurvedic medicinal plants were added along with the control solution for this study. This is discussed next considering the growth of urinary-type micro-crystals of CHPD (brushite) to demonstrate the potential application of this technique.

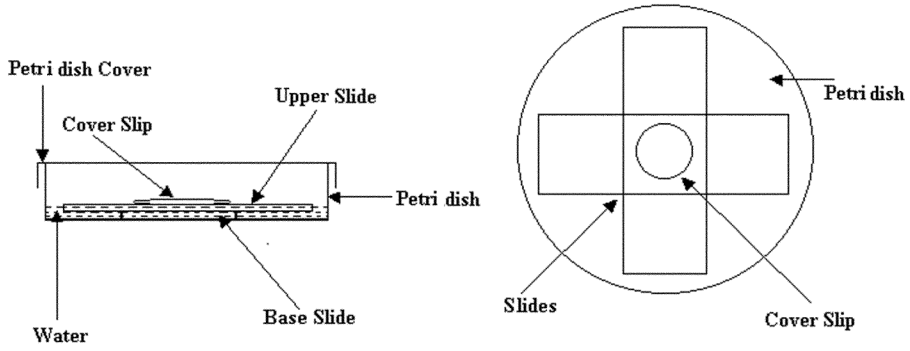

Figure 1. Modified gel growth method for micro-crystal growth.

Figure 2. Different morphologies of CHPD crystals. *a*, Platelet-type; *b*, Star-type with broad leaflets; *c*, Needle-type. Magnification 400×.

To grow micro-crystals of CHPD, a sodium metasilicate solution of specific gravity 1.06 was mixed with 1 M orthophospheric acid solution, so that pH 5.0 could be set for the mixture. The gel was set on the glass slides as mentioned earlier. Water was sucked from the petri dish with the help of glass dropper. Thereafter, 1 M $CaCl_2$ solution was poured with care using a dropper. The poured solution diffused through the gel and reacted with the already impregnated orthophospheric acid in the gel, which resulted in the nucleation of CHPD and, subsequently growth into micro-crystals within 24 h. Sodium metasilicate gel remained inert and allowed diffusion of reactants through its pores[22].

Rhombic platelet-type, star-type and needle-type crystal morphologies were mostly observed (Figure 2 *a*–*c*). The star-type and platelet-type crystals had maximum length of 60 and 40 μm, respectively. The crystals were observed using an optical microscope and photographs taken using CCD camera. The length of crystals was measured using image tool software.

The *in situ* observation of change in the morphology can be serially made. In the rhombic platelet, some further development of leaflets on its surface was observed (Figure 3). In star-type crystals, all needle-type or broad needle-type branches developed from a common centre, as shown in Figure 4 *a* and *b*. Possible formation of another needle by protruding parts on different sides of the needle can be seen in Figure 4 *a*. The leaflet-type of structure in Figure 3 is like mica sheets and getting new leaflets. This has been earlier observed by Joshi and Joshi[23] using SEM.

Growth inhibition or dissolution of crystals can be studied in the gel growth of micro-crystals. Many solutions, such as weak organic acids and herbal extracts of ayurvedic plants can be tested for this purpose. We have demonstrated this for CHPD crystals using citric acid solution. Citric acid is naturally available in many fruits[24,25] and various authors have already established citrate inhibition of urinary stones[26–28] and urinary-type crystals[18,21].

First the growth experiment of CHPD was conducted in several slides and, thereafter, solutions of 0.2, 0.4, 0.6, 0.8 and 1.0 molar concentration of citric acid were added in equal amounts using a micropipette to $CaCl_2$ solution in the respective petri dishes. The experiment was conducted at physiological temperatures in a controlled environment.

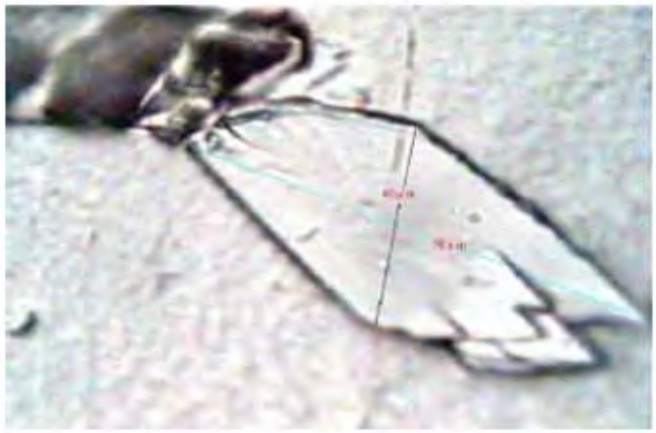

Figure 3. Further development of leaflets on the surface of the rhombic plate. Magnification 400×.

The growth of micro-crystals was monitored before and after adding citric acid solutions, under an optical microscope at regular time intervals. For comparison, in each set three petri dishes were used without adding any citric acid solution, i.e. they served as controls. Observations were made using Carl Zeiss (AXIOSKOP 2 plus) microscope in the transmitted light mode with the help of different filters and photographs taken using CCD camera attached to the microscope with maximum magnification 40×. The experiments were conducted in aseptic conditions to avoid microbial contamination. By placing a suitable mark on the left and right hand side of the cover slip on the slide and using the *X*–*Y* scale attached on the sample holder stage of the microscope, the location of the particular micro-crystal was found with reference to the marks. The proper microcrystalline sample was brought back for successive observations by repeating this, which can also be verified by the common features of the background.

In case of 0.2 M citric acid solution, crystals were observed after 2 and 12 h from the time of pouring the solution. The results are shown in Figure 5 *a* and *b* respectively. One can see that the crystal has been dissolved into a small fragment after 12 h. Figure 6 *a* indicates that the dissolution begins on a needle-type crystal within 2 h of pouring 0.4 M citric acid solution. Further, the same needle is fragmented after 12 h of pouring the solution (Figure 6 *b*). The gel is more structured because a significant portion

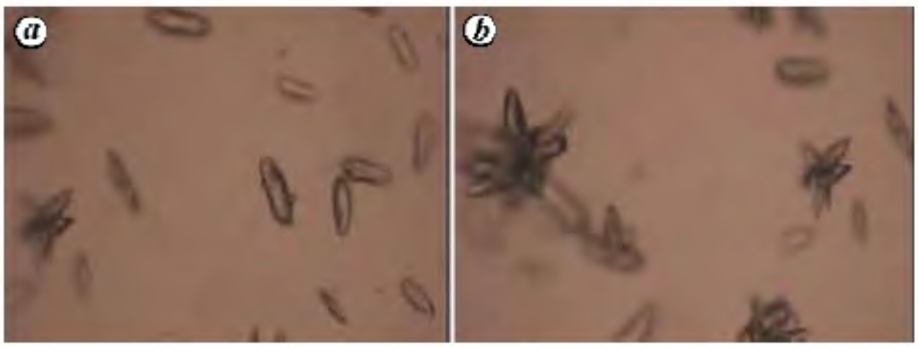

Figure 4. *a*, Possible formation of another branch of needle by protruding parts on different sides. *b*, Needle-type crystals originating from a common centre. Magnification 400×.

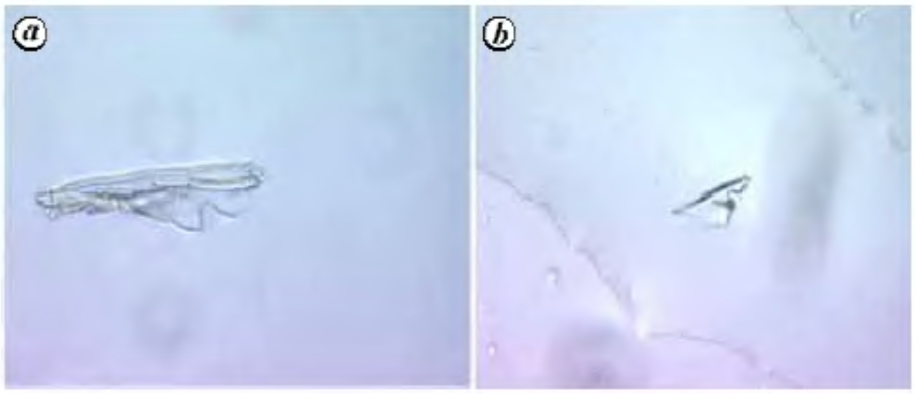

Figure 5. CHPD micro-crystals observed after (*a*) 2 h and (*b*) 12 h from pouring 0.2 M citric acid solution. Magnification 400×.

of the crystal has been dissolved and the solution collected during the process had spread to the surroundings through the porous medium of the gel. It has been observed that the dissolution of crystals was more rapid as the concentration of citric acid increased. This type of study can be carried out using various solutions and extracts of putative ayurvedic medicinal plants claimed to be useful in urolithiasis.

Attempts have been made to study the growth of several micro-crystals. Benzoic acid micro-crystals of 69–218 μm size have been obtained from saturated ethanol–water solution by adding water or an ethanol–water mixture in semi-batch experiments. The influence of size and shape was studied in different experiments[29]. Chayen[30] had highlighted oil-based technologies for producing crystals of biological macromolecules. In this technique, a crystallization drop was suspended between two oil samples; the bottom layer contained high-density oil and the top layer contained low-density oil. The drop had a density between that of the two oil samples and was at the interface between them. Micro-crystals have been obtained using micro-batch techniques.

Several sophisticated techniques have been developed for *in situ* crystallization. For instance, small angle and wide angle X-ray scattering techniques[31], atomic force microscopy[32] and holography[33]. However, these techniques are expensive and difficult to conduct in small laboratories. The common phenomenon of dissolution of a crystal can be tested by placing it in the selected solution and observing it at different time intervals. The growth of biomaterial crystals *in vivo* occurs with continuous supply of the accreting substrates; however, in that environment it is important to verify the growth dissolution or inhibition. In case the growth rate and dissolution rate become equal, growth of the crystal ceases. However, if the growth rate is more than the dissolution rate the inhibition phenomenon takes place, which can be confirmed by comparing the growth in pure control solutions without addition of inhibitors. Obviously, the growth of crystals is more in pure control solutions. When the dissolution rate is more than the growth rate, net dissolution is observed. Also, selective inhibition affects the particular crystallographic plane and modifies the morphology of a crystal, which depends on the crystal structure and type of molecules of the dissolving and inhibiting solutions as well as their molecular fit in the particular crystallographic face of the crystal[34]. Change in morphology is an important phenomenon to study. If the painful star-type or spiky urinary stones are converted into smooth spherical ones, then their passage through the urethra is less painful.

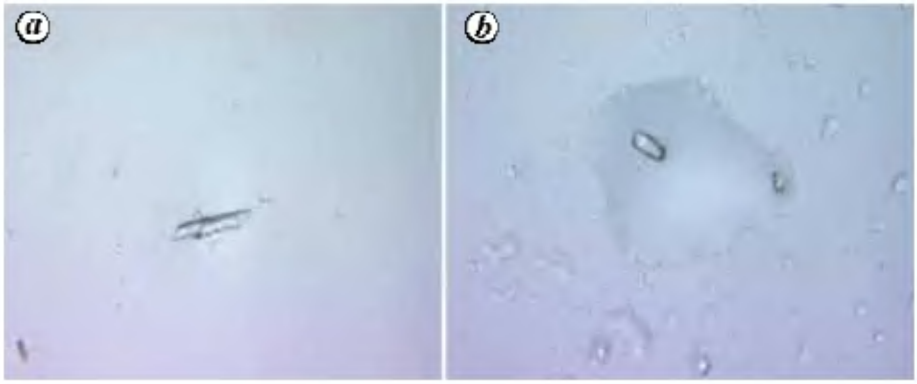

Figure 6. Dissolution of needle-type CHPD micro-crystal after (*a*) 2 h and (*b*) 12 h from pouring 0.4 M citric acid solution. Magnification 400×.

The current technique is comparatively inexpensive, rapid, requires less amount of material and solution, continuous growth or dissolution observations can be made depending upon the available facility (CCD camera, time lapse photography or movie recording) and large number of solutions or herbal extracts can be tested in aseptic condition.

Certain designing modifications can be introduced in the microscope for observations at physiological temperatures and in the batches of sample slides. For carrying out observations at physiological temperatures, certain modifications in the sample holder stage have been proposed. A small plate with electrical heating element connected to a temperature controller can be placed on the sample stage to heat the sample slides to physiological temperature. However, for measurement of batches of slides, the sample stage can be modified to accommodate several slides and can be moved by turning screws in the *X–X* direction precisely by marking on the scale.

In the pharmaceutical industry, growth, morphology and dissolution of crystals are important stages. In industrial processes, crystallization is an important operation which controls the physical properties of materials such as crystal habit, crystal size distribution and polymorphism. In the case of organic drugs which are slightly soluble in gastrointestinal fluids, dissolution rate of the solid dosage form is an important factor determining the rate of absorption. The kinetics of solubilization will depend mainly on crystal habit, crystal size distribution and polymorphism[35]. Microcrystal growth of pharmaceutical materials in gel medium is expected to provide, growth, dissolution, inhibition and morphological information for the purpose of drug designing. Crystal morphology is responsible for drug dissolution and activity, which can be studied for different morphologies of crystals using the present technique. Generally, control of morphology depends on many factors such as gel pH, gel density, concentration and type of solution used. Precise tuning in these factors can be reached after repeating experiments for different conditions for a particular morphology.

This method can be employed as a good screening technique to study the growth, dissolution, inhibition and morphological changes in micro-crystals. It can also be used as a good screening technique to identify the potent solutions which can dissolve or inhibit the crystals under growth conditions.

A modified gel growth technique has been proposed for the growth of micro-crystals of CHPD. *In situ* observations of growth of crystals and their morphologies are possible using an optical microscope.

An attempt has been made to study the growth inhibition and dissolution of CHPD micro-crystals using citric acid of different molar concentrations. Common morphologies of CHPD crystals such as rhombic platelets, needles and star-types have been observed. It has been found that as the concentration of citric acid increases, the crystal dissolution becomes more rapid. This confirms the earlier established citrate inhibition theory.

The proposed technique is inexpensive, needs less amount of material, and can be used for *in situ* growth inhibition and dissolution studies of biomaterial crystals. This technique, which mimics the growth of biomaterials in a body, is useful to clinical pharmacologists as a screening method to design therapies *in vitro*.

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ACKNOWLEDGEMENTS. We thank the Department of Biotechnology (DBT), New Delhi, for funds and Dr Vrinda S. Thaker, Bioscience Department, Saurashtra University, Rajkot, for experimental facilities. We also thank Dr Ashwinikumar Raut and Dr Sushma Mengi for their inputs and further work on the micro-crystals. B.B.P. thanks DBT for JRF/SRF.

Received 10 August 2006; revised accepted 3 April 2007

Rapid reversed-phase high performance liquid chromatography for vitexin analysis and fingerprint of *Passiflora foetida*

Narongchai Pongpan[1], Omboon Luanratana[1] and Leena Suntornsuk[2,*]

[1]Department of Pharmacognosy, and
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayudhaya Road, Rajathevee, Bangkok 10400, Thailand

Reversed phase high performance liquid chromatography (RP-HPLC) was developed for quantitation of vitexin and for examining the fingerprint of *Passiflora foetida* leaf extract. The simple isocratic condition consisting of isopropanol : tetrahydrofuran : water (5 : 15 : 80, v/v/v) and 0.3% formic acid with a flow rate of 1.3 ml/min was optimized. Under the optimum condition,

*For correspondence. (e-mail: pylll@mahidol.ac.th)