CHAPTER 2

CRYSTALLIZATION OF CHOLESTEROL

2.1 INTRODUCTION

There has been intensive study and considerable progress in the crystallization of biological stone minerals over the past few years (D alas and Koutsoukos 1989; Narayana Kalkura and Devanarayanan 1991; Wim Heijnen 1986). This is mainly due to the fact that a detailed knowledge of the nucleation and crystallization behaviour of biological crystals in laboratory conditions will throw light on the mechanism of nucleation and crystallization behaviour in vivo. Also it is advantageous to study the effect of additives that modify the nucleation and growth process in laboratory conditions which could be later extended to in vivo studies.

Cholesterol is a steroid, present in gallstones and atherosclerosis plaques. There have been a number of studies regarding the nucleation and growth of cholesterol in model bile (Holzbach et al 1984; Kibe et al 1985). But the studies are very preliminary in the sense that there is no conclusive alternative medicine to treat the gallstones and atherosclerosis without side effects. Although there has been considerable advances in allopathy treatment, there is still a need to go in for the system for which does not have any side effects and the disease is being easily cured. For example, Phyllanthus niruri is known to be the best native medicine for curing jaundice without any side effect. It is known for years that the Jain community from the northern part of India is very less prone to heart diseases and it has been found that they use more amount of garlic and
onion in their food. So far, not much work has been carried out to study the effect of inhibitor on the crystallization of cholesterol in vitro. In this chapter, the general principle of crystal growth in gel medium, the growth of cholesterol in the gel and the characterization of gel grown cholesterol crystals have been outlined.

2.2 METHODS OF CRYSTAL GROWTH

The selection of crystal growth technique is always made on the basis of the material characteristics, the growth kinetics and the requirements of the crystals such as size, shape and purity when more than one technique can be employed for growing single crystals of a given material.

Crystal growth involves careful control of a phase transformation. Based on this, the crystal growth methods can be broadly classified into four main categories:

1. Solid state growth ——> Solid to solid phase transition
2. Vapour growth ——> Vapour to solid phase transition
3. Melt growth ——> Liquid to solid phase transition
4. Solution growth ——> Liquid to solid phase transition

The classification of different growth techniques employed for growing crystals to meet specific demands in day-to-day life both in science and technology has been found in literature (Brice 1973; Brice 1986; Henisch 1988; Mullin 1961, Pamplin 1980). The first three types of crystal growth are not suitable for the growth of biological crystals and hence they are not discussed in this thesis. Gel growth is a particular case of solution growth and consequently ruled by similar growth parameters. However, some of the parameters can be varied much more conveniently in gel growth. Biological
crystals are mostly grown either in solution or in gel medium since the crystal growth conditions are similar to the biological conditions. Also the conditions such as pH, viscosity, temperature etc., can be altered in such a way that one can simulate conditions similar to that of the body fluids.

2.2.1 Gel

A gel is an interconnected network of solid with pores of submicrometer dimensions filled with liquid and the polymeric chains whose average length is greater than a micrometer. The term "gel" embraces a diversity of combinations of substances that can be classified into four categories as discussed by Hench and West (1990) as follows:

i) well-ordered lamellar structures
ii) covalent polymeric networks, completely disordered
iii) polymer networks formed through physical aggregation, predominantly disordered
iv) particular disordered structures.

2.3 BASIC CONSIDERATION OF GEL METHOD

The gels used for crystal growth are generally hydrogels. They are two component systems with the growth solution soaking a microporous flexible polymer network. The gelation process corresponds to the setting of a polymeric cluster stretching over the whole volume of solution. This process is either reversible for physical gels which are obtained by decreasing temperature or irreversible for chemical gels which are obtained by the formation of strong bonds. Although a universal 'best' gel cannot be recommended, silica gels and agar-agar gels have proved their efficiency for growing a number of crystals.
2.3.1 Types of gel

The gel can be classified as physical gel and chemical gel. The various types of gels used for crystal growth are given in Table 2.1.

a. Physical gel: This type of gel is obtained by physical process such as heating and cooling. Gelatin, agar agar and clay are examples of physical gels.

b. Chemical gel: Chemical gel is formed by chemical reactions such as hydrolysis or polymerization. Silica, polyacrylamide, tetramethoxysilane are examples of chemical gels.

2.3.2 Structure of gel

Gels are formed from suspensions or solutions by the establishment of a three dimensional system of cross-linkages between the molecules of one component. The second component (most commonly water) permeates the network as a continuous phase. A gel can thus be regarded as a loosely inter-linked polymer. The silica gel presents several advantages: it is stable and compatible with many substances because of its chemical composition close to glass.

2.3.3 Growth of crystals from gel

Among the variety of techniques prevalent today for the growth of single crystals, gel technique has gained considerable importance due to its simplicity and effectiveness in growing single crystals of certain compounds that cannot be easily be grown by other methods.
<table>
<thead>
<tr>
<th>Gel preparation</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Silica gel:</strong> Mix aqueous solution of sodium (meta) silicate (1.03-1.06 g/cc) and acid (1 to 4N of mineral or organic) or by hydrolysis of siloxanes like tetramethoxy silane or tetra ethoxy silane.</td>
<td>Better crystallinity</td>
<td>SiO₂ inclusion.</td>
</tr>
<tr>
<td><strong>2. Gelatin:</strong> Dissolve 5 to 6 g of gelatin powder in 100 ml of water by stirring at a constant temperature of 50°C for one hour and cool to room temperature. In order to strengthen the gel, addition of 0.1 to 1.0 ml of formaldehyde is necessary.</td>
<td>Great stability over large pH range (3 to 10.5).</td>
<td>Attacked by moulds.</td>
</tr>
<tr>
<td><strong>3. Agar:</strong> Dissolve 1 to 5 g of agar powder in 100 ml of water, boil it and cool to room temperature.</td>
<td>Easy work up.</td>
<td>Supports more nucleation sites.</td>
</tr>
<tr>
<td><strong>4. Clay (or Bentonite):</strong> Powdered clay slowly sifted in rapidly stirred water in a blender until about 9% clay had been added. Gel sets immediately.</td>
<td>Morphology of crystals grown is similar to those in many natural occurrences.</td>
<td>Crystals generally contains abundant kinks and pits.</td>
</tr>
<tr>
<td><strong>5. Polyacrylamide:</strong> Dissolve 3.99 wt% of acrylamide and 0.02 wt% of the cross linking agent in water, bubble with N₂, degas by reducing the pressure.</td>
<td>It is more rigid and transparent for more than six months in a wide range of pH from 0.2-13.</td>
<td>Preparation of gel is more difficult compared to others.</td>
</tr>
</tbody>
</table>
The gel medium prevents turbulence and helps the formation of good crystals by providing a frame work of nucleation sites. Another important advantage to be noted is that the convection current is totally absent in gel growth experiments. The reaction is mostly controlled by diffusion. Entrapping of growth solution by the gel network prevents the onset of natural convection which are density gradient induced movements of macroscopic volume of the liquid. These gradients are unavoidable in growth solutions because they correspond to driving forces for mass transfer to the growing interface. In gel media, mass transfer proceeds by diffusion through the gel pores which provides a regular and somewhat adjustable supply of solute. Hence a high degree of perfection and lesser number of defects have been observed in gel growth experiments. The gel method has also been applied to study the crystal formation in urinary calculi and in rheumatic diseases.

2.3.4 Importance of gel technique

In an ideal crystal every atom is surrounded by a uniform spatial arrangement of other atoms. If the arrangement of atom is not perfect, various kinds of imperfections are introduced. The physical properties like electrical, optical and mechanical properties of the crystals are controlled by these imperfections. Therefore, crystal growth involves not only preparation of single crystals but also control of the defect formation. The available literature and the review works clearly reveal the importance and the versatility of this growth technique. The following points emphasise the importance of the gel method:

* the gelation structure provides an ideal medium for the diffusion of reacting ions and can be used to keep the reacting ions separated until reaction is desired.
it is chemically inert and therefore, it doesn't react chemically with the reactants during crystallization.

it permits the reactant to diffuse through the gel within a controllable slow rate.

it retards convection during growth. Hence the formation of non-equilibrium defects is highly minimised.

concentration of the reactants can be easily varied.

nucleation can be controlled by varying the density of the gel as well as by varying the concentration of the reactants and the growth at each stage can be observed through the transparent medium.

the grown crystals can be harvested easily without damaging the crystal faces. Crystals grown by this technique are found to have less dislocations than the crystals grown by other methods.

all nuclei are spatially separated, minimizing the precipitation.

crystals with different morphologies and sizes can be obtained by changing the growth conditions

this method is extremely simple and inexpensive.

for biological materials, gel method is the best option since one can simulate the body conditions.
the gel method is a best method for the macromolecular crystal growth since it avoids convection and also smaller amount of source material is sufficient.

2.4 TYPES OF GEL GROWTH METHODS

A useful survey of growth procedure has been provided by Henisch (1988) and by Patel and Venkateswara Rao (1982). The gel growth methods are classified into the following three methods:

1. Chemical reaction method
2. Solubility reduction method and
3. Complex - decomplexing method

Depending upon the technique used, the material to be grown and the inference desired from the techniques, one can employ either single diffusion or double diffusion methods.

2.4.1 Chemical reaction method

Two aqueous solutions of soluble salts are suitably chosen and then allowed to diffuse through a gel from both sides of the gel medium. These reactants diffuse through and meet at some place and react to yield the reaction product. When the reaction product exceeds the saturation limit, nucleations occur and grow into single crystals. Consider a general case, where AX and BY are the solutions of two compounds which on reaction give rise to the insoluble or sparingly soluble product AB and also the highly soluble waste product XY. i.e.,

\[ AX + BY \rightarrow AB + XY \]
There are basically four ways to set up the experimental arrangement to grow single crystals by chemical reaction method. Figs.2.1a-d illustrate these four types.

The simplest method (Fig.2.1a) is that one of the reactants is taken along with the gel solution and set into a gel in a straight tube. After gelation, the other reactant is taken over the gel as outer reactant. The outer reactant diffuses into the gel and reacts with the inner reactant. When the concentration of the reaction product exceeds the solubility product, nucleations are formed and then these nuclei grow into crystals of larger size. Single crystals like PbI₂, CaCO₃ are grown by this method.

Fig.2.1b shows an extension of the method discussed in Fig.2.1a. Here a neutral gel column is introduced over the set gel and over the neutral gel the outer reactant is taken. The introduction of neutral gel, reduces the nucleation density and hence the size of the crystals can be increased.

Fig.2.1c shows a technique where the gel is set over the packed reactant taken at the bottom of a straight tube. The other reactant is taken over the set gel. Both the reactants diffuse towards each other and meet at a plane in the gel. Reaction takes place in the vicinity of this plane and the crystals grow in that region.

In the fourth type, a neutral gel is set in the U-tube (Fig. 2.1d). The two reactants (AX and BY) are taken on either sides of the limbs. The reactants on diffusion form the crystals in the bent portion of the U tube. The diffusion involved may be either single diffusion or double diffusion. Most of the work on crystal growth in gels has been carried out by this method. The necessary conditions required to grow single crystals by this method are:
Figure 2.1a Straight tube method with reactant-1 taken with gel.

Figure 2.1b Straight tube method with reactant-1 taken in a bag.
Figure 2.1c Straight tube method with neutral gel.

Figure 2.1d U-tube method.
the reactants employed here must be soluble in the solvent (usually water) and the reaction product of interest must be less soluble whereas the other reaction product, usually known as waste product should be highly soluble.

the gel must remain stable in the presence of the reacting solutions and must not react with the reactants and the products formed.

2.4.2 Complex-decomplexing method

The material to be crystallized is first made to form a complex by means of suitable reagent and then allowed to diffuse into the gel, free of active reagents. Decomplexing starts with increasing dilution and leads to the high supersaturations necessary for crystal growth. For eg., silver iodide has been grown by complexing it with potassium iodide. The high solubility of silver iodide in concentrated potassium iodide solution and its rapid decrease in solubility with dilution of potassium iodide solution makes this method particularly suitable. The decomplexing procedure is a versatile and valuable addition to the repertoire of gel growth methods and the geometry employed permits the removal of waste products in a simple and continuous way.

2.4.3 Reduction of solubility method

It is possible to grow single crystals of materials of high solubility by reducing its solubility using suitable reactants. Triglycine sulfate, highly soluble in water, but much less soluble in alcohol is grown by this method. A gel is made by mixing sodium meta silicate solution, glycine and sulfuric acid in appropriate concentration and quantities. Supernatent ethanol is allowed to diffuse into this gel after setting which produces bulky crystals
and needles of triglycine sulfate, often with excellent surfaces and optical clarity. Crystals of KDP, ADP are grown by this method.

2.5 CRYSTALLIZATION OF CHOLESTEROL

Cholesterol has been crystallized in silica gel medium using solubility reduction technique. Gel is prepared by dissolving sodium meta silicate (Loba, chemie) in double distilled water to the required density. The density of the gel solution has been varied from 1.03 g/cc to 1.06 g/cc. The pH of the solution to the required value is adjusted by titrating this solution with glacial acetic acid. The pH of the gel solution has been varied from 3.0 to 6.0. Depending upon the concentration and the pH of the gel medium the time of gelation varies from one day to three days. The supernatent solution is prepared by dissolving cholesterol(SRL) in acetone (AR, Emerck), ethanol (AR, Emerck) and isopropanol (AR, Emerck) to the required concentration. The concentration of the supernatent solution was varied from 0.25% to 1.0% (w/v). It was found that when the pH value was less than 4.0 and the density of the SMS solution was 1.03 g/cc the resulting gel was unstable. When the pH value was higher than 5.5 and density of the SMS solution was 1.06 g/cc the resulting gel was opaque. After the gelation, the supernatent solution was poured gently on the top of the gel medium. Since cholesterol is sparingly soluble in water, as it diffuses into the gel medium, the solubility of the cholesterol in the solvent reduced and hence due to the supersaturation the crystals are formed. It took approximately one to three days for the nucleation to be observed visually.

The nucleation and final size of the grown crystals depend on the pH of the medium, concentration of the supernatent solution and density of the gel solution. The morphologies (platy, needles) are independent of the pH, density of the gel and the concentration of the supernatent solution. The crystals are viewed for their size measurement with the use of optical
When cholesterol dissolved in acetone or isopropanol was used as the supernatent solution, a ring pattern of platy and needle crystals was observed initially. These needle crystals slowly disappeared in a period of two months and platy crystals formed. Initial trial experiments were carried out to grow cholesterol crystals in gel medium using cholesterol dissolved in acetone, ethanol, acetic acid and isopropanol as the supernatent solution. Cholesterol dissolved in isopropanol has been used as the supernatent solution to study the effect of pH, density of the gel and the concentration of the supernatent solution.

The grown crystals were characterised using XRD, IR and thermal analysis. The IR spectra was taken using Perkin Elmer 397 spectrometer by the KBr pellet technique. The thermal analysis was carried out using Perkin Elmer DSC 7. XRD studies were carried out using RICH SEIFERT x-ray powder diffractometer.

2.6 RESULTS AND DISCUSSION

2.6.1 Crystallization of cholesterol

The SMS solution of density 1.06 g/cc, pH 4 and supernatent solution of concentration 0.5%(w/v) was used as the reference (control) to study the effect of medicinal plants on the growth of cholesterol. In control, the crystals were found to grow in a day after the addition of supernatent solution and the growth is completed in a period of 4 months. Also two rings of width 2.0 mm containing platy crystals at a distance of 5.0 mm and 8.0 mm from the interface were formed in three days. Beyond the rings, one ring of needle crystals also found to grow. Within a period of one week, the size of the platy crystals varied from 0.06 * 0.2 mm to 0.06 * 0.3 mm and the length of the needles was approximately 0.04 mm in three days. After 45 days these needles grow upto 1.0 mm in the needle axis and slowly
changes into platy crystals due to Ostwald’s ripening. After three months, the maximum size of the platy crystals varied from $0.1 \times 0.2$ mm to a maximum of $0.4 \times 0.6$ mm.

2.6.2 Morphology

When cholesterol dissolved in acetone was used as the supernatent solution, fibrous needle crystals (Fig.2.2) were found to grow inside the gel medium at the initial stage. After 45 days these fibrous crystals slowly transformed into platy crystals. When the cholesterol dissolved in ethanol was used as the supernatent solution, thin platy crystals (Fig.2.3) appeared inside the gel medium. When the cholesterol dissolved in glacial acetic acid was used as the supernatent solution lengthy needle crystals (Fig.2.4) were found to grow. When the cholesterol dissolved in isopropanol was used as the supernatent solution, needle (Fig.2.5) and platy (Fig.2.6) crystals were formed initially. The needle crystals were transformed into platy crystals at the final stage.

2.6.3 Effect of pH of the gel medium

Fig.2.7 shows the crystals grown in the gel medium of density 1.05 g/cc and pH values of 4, 4.6 and 5.2 respectively with 0.25% (w/v) solution of cholesterol in isopropanol as the supernatent solution. The number of crystals decreases and the size of the crystals increases as the pH value decreases. They are represented by the tubes marked as a, b and c respectively.

2.6.4 Effect of density of gel

Fig.2.8 shows the crystals grown for various densities of the gel (1.04, 1.05 and 1.06 g/cc) at the pH value of 5.2 and 0.25% (w/v) solution of
cholesterol in isopropanol as the supernatent solution. Here the first ring contains more number of crystals for the gel of density 1.04 g/cc than the higher density gel. They are represented by tubes marked as d, e and f respectively.

2.6.5 Effect of concentration of the supernatent solution

Fig.2.9 shows the crystals grown in the gel medium of density 1.05 g/cc and pH value of 5.2 for various concentrations of supernatent solution (0.25, 0.5 and 1.0% (w/v) of cholesterol in isopropanol. They are represented by the tubes g, h and i in the Fig.2.9.

When 1.0% solution of cholesterol in isopropanol was used as the supernatent solution, fibrous cholesterol crystals were observed in the supernatent solution itself. The formation of crystal nuclei is also nearer to the interface when compared to the crystallization at 0.25% supernatent solution.

2.6.6 Infrared analysis

Fig.2.10. represents the IR spectrum of cholesterol grown in the gel. The absorption peaks are compared with the literature values (Dalas and Koutsoukos 1989). The broad envelope lying between 2600 and 3600 cm\(^{-1}\) includes the stretching modes due to O-H (3600 - 3500 cm\(^{-1}\)), olefinic C-H (3010 cm\(^{-1}\)) and alkyl C-H (2986, 2950 cm\(^{-1}\)) bonds. As the envelope extends well below 3000 cm\(^{-1}\), the molecular association in the crystal would be strongly hydrogen bond dependent through alcoholic O-H group. Although alkyl residues are available in addition to ring CH grouping, their C-H stretch modes are not resolved and hence their contribution to crystal packing through weak Van der waals interaction would not be largely significant. CO stretch (1010 cm\(^{-1}\)) is also broadened supporting hydrogen
Figure 2.10 IR spectrum of cholesterol.
bonded interactions between OH groups. The bands due to C-H in plane bend (1261 cm\(^{-1}\)) and its out of plane bend (1185 cm\(^{-1}\)) get mixed up with C-O stretch mode. Asymmetric (1450 cm\(^{-1}\)) and symmetric (1375 cm\(^{-1}\)) bends of CH are positioned above and just below 1400 cm\(^{-1}\). C=C stretch is clearly evident around 1620 cm\(^{-1}\), methylene rock is placed at 750 cm\(^{-1}\). C-OH inplane bends positioned below 600 cm\(^{-1}\) are well resolved with broadening. The observed vibrational frequencies and their assignments are listed in Table 2.2.

### 2.6.7 Thermal analysis

The DSC profile is represented in Fig.2.11 and agrees well with the literature (Loomis et al 1979). Three endotherms observed at 80, 124 and 152°C confirm that the grown crystals were cholesterol monohydrate. The transition observed by DSC at 80°C represents the transformation of cholesterol monohydrate to the high temperature anhydrous cholesterol polymorph with the loss of water on hydration. A sharp transition at 124°C is representing a crystalline to liquid crystalline transition of cholesterol. On further heating the liquid crystalline phase melted at 152°C.

### 2.6.8 X-ray diffraction

The XRD pattern of the grown cholesterol crystals is shown in Fig.2.12 and the experimental data match well with the literature values (Garti et al 1981). The details of the X-ray powder analysis are given in Table 2.3.
<table>
<thead>
<tr>
<th>Wave number (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3470</td>
<td>OH stretch</td>
</tr>
<tr>
<td>1635</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1600</td>
<td></td>
</tr>
<tr>
<td>1550</td>
<td></td>
</tr>
<tr>
<td>1440</td>
<td>Ring stretch</td>
</tr>
<tr>
<td>1410</td>
<td></td>
</tr>
<tr>
<td>1120</td>
<td>C-C-C inplane bend</td>
</tr>
<tr>
<td>1030</td>
<td>C-H out of plane bend</td>
</tr>
<tr>
<td>870</td>
<td>C-C-C stretching</td>
</tr>
<tr>
<td>790</td>
<td>Skeletal distortion</td>
</tr>
<tr>
<td>670</td>
<td></td>
</tr>
<tr>
<td>595</td>
<td>C-OH inplane bend</td>
</tr>
<tr>
<td>555</td>
<td>C=O inplane bend</td>
</tr>
</tbody>
</table>
Figure 2.11 DSC heating scan of cholesterol
Figure 2.12 Powder x-ray diffraction pattern of cholesterol.
TABLE 2.3 X-RAY DIFFRACTION DATA OF GEL GROWN CHOLESTEROL CRYSTAL

<table>
<thead>
<tr>
<th>2θ</th>
<th>Measured $d_{hkl}$(nm)</th>
<th>Calculated $d_{hkl}$(nm)</th>
<th>(hkl)</th>
</tr>
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<tbody>
<tr>
<td>23.7</td>
<td>0.375</td>
<td>0.375</td>
<td>224</td>
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<tr>
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<td>0.409</td>
<td>0.409</td>
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<tr>
<td>19.6</td>
<td>0.453</td>
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</tr>
<tr>
<td>19.1</td>
<td>0.465</td>
<td>0.467</td>
<td>221</td>
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<tr>
<td>18.4</td>
<td>0.484</td>
<td>0.487</td>
<td>211</td>
</tr>
<tr>
<td>17.5</td>
<td>0.507</td>
<td>0.508</td>
<td>121</td>
</tr>
<tr>
<td>17.2</td>
<td>0.517</td>
<td>0.521</td>
<td>123</td>
</tr>
<tr>
<td>15.6</td>
<td>0.568</td>
<td>0.568</td>
<td>203</td>
</tr>
<tr>
<td>14.4</td>
<td>0.618</td>
<td>0.613</td>
<td>114</td>
</tr>
<tr>
<td>13.3</td>
<td>0.666</td>
<td>0.677</td>
<td>112</td>
</tr>
<tr>
<td>11.9</td>
<td>0.763</td>
<td>0.770</td>
<td>103</td>
</tr>
<tr>
<td>10.8</td>
<td>0.820</td>
<td>0.807</td>
<td>112</td>
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</tbody>
</table>
2.7 CONCLUSION

Single crystals of cholesterol have been grown in sodium silicate gel. The number density and the size of the crystals depend on the density of the gel, pH of the gel and the concentration of the supernatent solution. The morphology of the crystals is independent of the above factors.