HOW DOES BOVINE SERUM ALBUMIN PREVENT THE FORMATION OF KIDNEY STONE? --- A KINETICS STUDY

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NATIONAL UNIVERSITY OF SINGAPORE

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THE FORMATION OF KIDNEY STONE? ---
A KINETICS STUDY

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I would like to express my sincere thanks to those who have helped and inspired me during the past two and half years of my study.

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**SUMMARY**

Calcium oxalate monohydrate is the main inorganic constituent of kidney stones. Thus, the study of calcium oxalate (CaOx) crystal formation is of major importance for human health. Urinary proteins are believed to have the potential to influence the crystallization of CaOx. Some papers have reported that the protein, albumin, promotes the nucleation of CaOx crystal by templating effect. However, others reported that this protein inhibited the formation of CaOx crystal. Therefore, how does the albumin affect the crystallization of urinary stone is still unclear.

Although some aspects of nucleation and aggregation of CaOx crystals in vitro have been studied including the effect of some human proteins, no detailed studies on the crystallization of CaOx crystals have been reported to elucidate the effect of these proteins. Evidently, an unambiguous understanding of the effects of these proteins on the formation of CaOx should be developed.

Recently, the structural synergy between biominerals and biosubstrates was examined. Particular emphasis was placed on the templating effect of the substrate, as well as a newly identified supersaturation-driven interfacial structure mismatch effect in the context of a new nucleation model. Based on this model, some exciting results have been achieved in studying ice, calcium carbonate and hydroxyapatite, through a comparative analysis of the effects of various selected additives (salts, and biopolymers). To obtain a better understanding on the CaOx crystallization and the role of the albumin in the urine, in this work, we employ the mentioned nucleation model, to examine the nucleation of Calcium Oxalate Monohydrate and the impact of
bovine serum albumin (BSA). In addition, we also examine how the BSA influences the assembly of CaOx from the kinetics point of view.

In this study, the influence of the BSA on the nucleation kinetics is discussed. First, the presence of BSA lowers the nucleation energy barrier. Second, during the nucleation process, the BSA adheres to the kink sites and/or the embryo surfaces; thus, the BSA increases the kink energy barrier, and slows down the crystallization. In essence, the BSA prolongs the CaOx nucleation process. This is accompanied by the increase in nucleation induction time. From the nucleation kinetics study, we also deduce that the protein can enlarge the supersaturation range to achieve a better crystal assembly. In addition, this conclusion has been confirmed by the crystal morphology study.

Since the BSA favors the formation of Calcium Oxalate Dihydrate (COD) crystal, we also discuss the possible role of the albumin in treating the kidney stone. As COD is less likely to adhere to the urinary cells and tubes, and it is less harmful to the kidney. Moreover, the induction time increase makes the crystals more easily propelled out by urine. These factors lead to the conclusion that the albumin plays a positive effect on preventing the kidney stone disease.

Though some progress has been made in our study on the kidney stone and the role of protein, this study has also put forward many questions, which still need satisfactory answers. I hope that these results would promote further study of the role of albumin on the CaOx crystal crystallization leading to an effective approach to control the formation of CaOx crystals, and contribute to the treatment of kidney stones.
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<td>Calcium Oxalate</td>
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CHAPTER ONE

Introduction

1.1 General Introduction of Biomineralization

The controlled formation of inorganic minerals in organisms results in the biomineralization of crystalline and amorphous materials\textsuperscript{1-8}. Mineralization processes, which are under strict biological control, are aimed at specific biological functions such as structural support\textsuperscript{6, 9} (bones and shells), mechanical strength\textsuperscript{7, 10, 11} (teeth), iron storage (ferritin) and magnetic\textsuperscript{5} and gravity reception\textsuperscript{12-14} etc. Studies of chemical and biochemical process of biomineralization not only lead to new insights in bioinorganic chemistry, but also provide novel concepts in crystal engineering and materials science.

The subject of biominerals covers a wide range of inorganic salts, which serve a variety of functions in biology. The field of biomineralization\textsuperscript{1-3, 12, 15-17} covers all phenomena that involve mineral formation by organisms. This includes the string of 50-nm-long magnetite\textsuperscript{5} crystals formed intracellularly by some bacteria, the two crystal specula skeleton of the larvae of sea urchins\textsuperscript{18}, and the huge molars and bones of elephants\textsuperscript{19}. We learn that biominerals are “smart” in that they are designed in response to external signals\textsuperscript{5}. Their functions are almost as varied\textsuperscript{2, 3, 5, 16, 17}: sound reception, gravity perception, toxic waste disposal, orientation in the earth’s magnetic
field, temporary storage of ions, and a diverse array of materials that are stiffened and hardened by the presence of mineral. There are many examples\textsuperscript{2, 3, 16, 17} of the control of form and microstructure for a mechanical duty. The antler bone of the deer is used in fighting and hence has high work of fracture for impact strength. The femur of a large animal such as a cow needs to support weight and is stiff with adequate toughness. In fact, there are also a great many other examples.

The body of biomineralization is huge as it covers a large scale of academic field for investigation\textsuperscript{4, 8, 20-25}. The materials used include more than 60 different mineral types, an array of structural proteins and polysaccharides, and many dedicated glycoproteins, whose major functions are to control in one way or another the mineralization process. The most basic processes in biomineralization operate at the nanometer length scales and involve proteins and/or other macromolecules directly in controlling the nucleation, growth, and promotion/inhibition of the mineral phase\textsuperscript{8, 24, 26}.

Many questions remain to be answered: How can such elaborate inorganic forms be sculptured by soft biological structures and systems? In addition, what role does structural biology play in the evolution of inorganic morphogenesis? One teasing question is whether any of the mineralization mechanisms operating in these invertebrates are precursors or even analogs to the large-scale structures of vertebrate mineralization, which not surprisingly are the most actively investigated of all biominerals.

The important applications of biomineralization and the need for increased activity among structural biologists in this field have attracted much of attention. Clearly,
biomineralized tissues such as bones and teeth continue to be of fundamental importance in medicine and health care. There are also other important implications of biomineralization research for new advances in materials science. For example, there is a growing interest in the use of biomineralization proteins and their synthetic analogues for the control of crystal properties and organization. These may lead to a rethinking of the formation and value of minerals, especially composites in industry. It is very likely that biomolecules will be used as templates for the fabrication of inorganic systems such as electronic devices, new catalysts, sensors, and porous materials, as well as biomimetic structures for more conventional uses in biomaterials. In each case, knowledge of the underlying biological structures is the basis for all novel applications.

1.2 General Introduction of Calcium Oxalate Crystal

Calcium oxalate\textsuperscript{24, 27-37} (CaOx) is quite common in nature and is found in almost all types of living beings, micro-organism, fungi, plants and animals including humans. In plants\textsuperscript{27} where a majority of the families of seed plants contain CaOx crystal deposits, it plays diverse roles such as storing excess calcium, forming exoskeleton or making plants less palatable to foraging animals. CaOx crystal can be found in all major groups of photosynthetic organisms\textsuperscript{24, 27, 28} including algae, lower vascular plants, gymnosperms, and angiosperms. CaOx crystal is also found in animals but in contrast to plants it is most commonly associated with the pathological condition of renal stone disease, although it occurs as a structural element in a few animals and as a potential defense in others\textsuperscript{28, 38}. 
In man and other mammals, oxalate is endogenously produced as well as obtained from the food. Since it cannot be metabolized, oxalate is excreted in the urine\textsuperscript{35, 39-41}. Urinary overexcretion of oxalate may result in crystal deposition in the kidneys, formation of kidney stones and eventually in renal failure\textsuperscript{42}. A number of people suffer from problems due to urinary stones (calculi). Areas of high incidence of urinary calculi include the British Isles, Scandinavian countries, northern Australia, Central Europe, northern India, Pakistan and Mediterranean countries. Saurashtra region, Gujarat has higher prevalence of urinary stones\textsuperscript{29}. According to an estimate, every year 600,000 Americans suffer from urinary stones. And, the cost of treating human urinary stone disease in the United States alone is estimated to be more 2.4 billion dollars per year\textsuperscript{28}. In India, 12\% of the population is expected to have urinary stones, out of which 50\% may end up with loss of kidneys or renal damage. In human\textsuperscript{35, 39-41, 43-46}, calcium stones are most common, comprising 75\% of all urinary calculi. Majority of them are calcium oxalate monohydrate (COM) whewellite or calcium oxalate dihydrate (COD) weddelite. In general, the urinary calculi are composed mainly of crystalline components. Thus, CaOx crystal is of major biological and economic importance.

The study of urinary stone and CaOx crystal is a rather complicated process. A combination of factors (gene and environment) play a role in defining CaOx crystal amount, shape, and size and thus function\textsuperscript{24, 27, 28}. Stone formation requires supersaturated urine, which depends on urinary pH, ionic strength, solute concentration and complexation. Knowledge of the processes involved in CaOx crystal formation is relevant to our basic understanding of organs, and specialized defense mechanisms. Studies on CaOx crystal formation and its regulation have also
provided insights into the fascinating large fluxes of Ca across multiple compartments, and for controlling CaOx crystal precipitation so that crystal growth does not cause unwanted damaged to cells. Considering the complexity of crystal formation, regulation can occur at a number of steps.

The major components of CaOx crystals are simple, but the resulting crystals can be complex in their morphology. Oxalic acid ($C_2H_2O_4$) is a strong organic acid with dissociation constants $P_{k_1} = 1.46$ and $P_{k_2} = 4.40$. Oxalic acid can complex with Ca to form highly insoluble CaOx crystals (solubility product, $K_{sp}$, at $25^\circ C$ of $2.32 \times 10^{-9}$ for the monohydrate$^{27}$) with a striking range of morphologies. To form a CaOx crystal, the agents in the environment can act as heterogeneous nucleates to lower the metastable limit and promote crystal formation$^{10, 47-49}$. Various charged compounds, including organic acids, peptides, polysaccharides, proteins, and lipids, have nucleation promoting or inhibiting properties in vitro. These compounds can change the physic-chemical dynamics and can affect the rate of formation, hydration state, morphology, and aggregation of crystals. Thus, although the chemistry of CaOx crystal precipitation is relatively simple, the addition of organic materials in the biological system complicates our understanding of the precipitation process.

### 1.3 Epidemiology of Calcium Oxalate Urolithiasis in Man

CaOx crystallization in vitro is usually carried out in the context of investigating urolithiasis$^{29, 33, 34, 39, 50-53}$. Applications range from studying fundamental physical chemistry in simple solutions to developing clinically meaningful tests using urine. In
the United State alone, urolithiasis accounts for approximately 200,000 hospitalizations per year. The incidence of urolithiasis has been increasing steadily in industrialized regions of the world since last century. CaOx crystal is by far the most common constituent of upper urinary tract calculi and may be important in endemic bladder calculi as well.

Some of the biologic factors that can influence the epidemiology of urolithiasis have been investigated:

1. The adult males are more likely to have symptomatic stones. In industrialized societies the urolithiasis occurs predominantly in mid adulthood with a much lower incidence in childhood and in the elderly.

2. There has been general agreement that blacks have a significantly lower prevalence of urolithiasis than whites. And, it is believed that the environmental factors that result in this race difference.

3. Individuals who have a family history of urolithiasis are more likely to form urinary tract stones than non-stone formers. Among stone patients with frequent recurrences, the likelihood of a positive family history is even higher.

4. It is known that diets low in animal protein and phosphorus and high in cereals favor the formation of endemic urinary stones, particularly in children. A diet rich in fiber may inhibit intestinal calcium absorption but may also facilitate absorption of oxalate. Finally, the water intake is also an important factor, for a man with the urine volume of less than one liter per day, the risk of nucleation of constituents leading to calcium stones rises dramatically.
As mentioned above, CaOx crystal formation is a fundamental part of the physiology of many species. Through the integration of ultrastructural, physiological, biochemical, and genetic approaches, the mechanisms responsible for this remarkable biomineralization process is being identified; however many features of crystal formation remain to be characterized. Thus, a better understanding of the mechanisms operating in CaOx crystal nucleation, growth and crystallization is needed to clearly characterize those features working in crystal formation, so as to solve those questions mentioned before and improve the urolithiasis treatment.

1.4 Objective of This Thesis

One of the reasons why Biomineralization is so important is its potential application in the medical field. Although, recently, a lot of work has been done on the urinary stone study, and some tremendous progress has been achieved, the influence of the proteins on the formation of urinary stone is still unclear. Tremendous work has deliberately been performed to contribute towards the purpose, namely, the exact role of the urinary protein in the urinary stone nucleation, growth and aggregation. These results are somewhat confusing due to the conflicting role of the protein predicted. This situation demands more concrete data and reasonable interpretation. Until now, it is well known that each protein plays its distinguishable part, but what kind of consequence and how the protein contributes to this is the hot debated issue.

As for the albumin, some papers have reported that it promotes the nucleation of CaOx crystal, the major component of urinary stone, by templating effect. However,
others reported experiments provided opposite results that albumin inhibits the formation of CaOx crystal. These conflicts\textsuperscript{15, 35, 37, 44, 51, 63} may arise from the experiment methods, but it will never be so simple to resolve them. How does the albumin affect the crystallization of urinary stone is still unknown.

To answer the questions mentioned above, this study is aimed at the investigation of how the protein, Bovine Serum Albumin, influences the nucleation of CaOx crystal, and the consequent crystal growth and aggregation. We notice that a newly formed nucleation theory that has been widely used on the nucleation of ice, CaCO\textsubscript{3} and hydroxyapatite has contributed a lot to the crystal study. So, it has been employed here on the nucleation study of CaOx crystal. As this work is mainly focused on how the proteins influence the crystallization, the templating effect of protein is also discussed. This study is also aimed to explain how the proteins lower the nucleation energy barrier, increase the kink site energy barrier and their potential role in inhibiting the formation of CaOx crystal. Lastly, this study intends to investigate the crystal morphology change produced by the presence of bovine serum albumin (BSA).

We wish that these results could promote the study of the role of albumin on the CaOx crystal crystallization and urinary stone formation. We also wish that this thesis could contribute towards research on the protein effect in the biomineralization world. The task is immense, but the future is bright.
1.5 Organization of This Thesis

This thesis is composed of six chapters, which include introduction, literature review, experiments, results, discussion and conclusion. The contents of each chapter are briefly given below.

The general knowledge on biomineralization is briefly introduced in the first chapter. The role of urinary stone to human health and related studies are also briefly listed.

The second chapter contains the literature review of the general nucleation knowledge and theory, which are used as foundation in this study. In this chapter, a newly founded nucleation theory is also introduced and discussed. The recent progress on urinary proteins and their influence on urinary stone formation are also presented.

The third chapter describes the techniques used in this study, which include Dynamic Light-Scattering system, X-ray diffraction (XRD), High Performance Particle Sizer (HPPS), Scanning Electron Microscope (SEM) and Zetasizer. Finally, the chemicals reagents used and some related information are listed.

At the beginning of the fourth chapter, the XRD experiment, which is used to confirm the crystals prepared in this study is discussed. Then the CaOx crystal nucleation kinetics with the effect of sodium chloride and the protein, bovine serum albumin, is examined. In this part, armed with the newly identified nucleation theory, the nucleation kinetics is carefully examined and discussed in detail. How the albumin influences the CaOx crystal nucleation process is also carefully discussed.
The fifth chapter mainly focuses on the CaOx crystal morphology study. The SEM pictures of the crystals are examined and how the protein, BSA, influence the morphology of CaOx crystal is discussed. These results mainly serve to confirm the conclusions deduced from the previous chapter.

Results reported in the preceding chapters are summarized in the last chapter: chapter six and the potential advantage of albumin in alleviating the urinary stone disease is also clarified. Major conclusions are drawn and recommendations on future work are given in this chapter.
CHAPTER TWO

Literature Review

2.1 Nucleation Theory

2.1.1 Introduction of General Nucleation Theory

The general nucleation process can be described as that\textsuperscript{2, 3, 10, 48, 49, 64, 65} by which the constituent units (molecules or ions) in the solution may, on collision, join into groups of two or more particles to form dimers, trimers, tetramers, and so forth. However, even when a positive thermodynamic driving force\textsuperscript{2, 3, 47, 64-66}, $\Delta \mu$, is acting on the embryos, they are still unstable, until the embryos can reach a critical radius, $r_c$. To reach the $r_c$, an energy barrier, the so-called nucleation barrier, needs to be overcome. During nucleation process, can the embryos reach the critical radius is the main concern\textsuperscript{2, 3, 47}. Once the nucleation barrier is overcome, the embryos can grow\textsuperscript{2, 3, 67}, thus the embryo enters the second step of phase transition: growth.

If nuclei are formed in perfectly clean solution in the absence of any foreign particles or surfaces, the nucleation mechanism is referred as “homogeneous” nucleation\textsuperscript{3, 67}, also sometimes called spontaneous nucleation. But in practical situation, the presence of foreign surfaces (in the form of ions, impurity molecules, dust particles, or other
surfaces) generally induces “heterogeneous” rather than homogeneous nucleation. The heterogeneous nucleation can occur at lower supersaturation than the homogeneous nucleation\textsuperscript{67}. Both these nucleation processes are forms of primary nucleation, so called to distinguish them from the second main category, secondary nucleation. It occurs only because of the prior presence of crystals of the material being crystallized. The classification\textsuperscript{2, 3, 67} of nucleation phenomena is shown in Table 2-1.

Crystals will not grow out of all supersaturated solutions. To create a new phase, the system must overcome a certain energy barrier called Gibbs Free energy, $\Delta G$. The occurrence of nucleation barrier is attributed to the following two-conservancy effects\textsuperscript{2, 3, 12, 47, 67}:

1. Since the crystalline phase is stable, the occurrence of the new phase from the mother phase will lead to the lowering of the (Gibbs) free energy of the system;

2. Due to the interfacial (or surface) free energy, the increase in the size of the crystalline (new) phase leads to the increase of interface (or surface) area,

\begin{center}
\includegraphics[width=\textwidth]{nucleation_diagram.png}
\end{center}

Table 2-1. Classification of nucleation phenomena
consequently causes the increase of the Gibbs free energy of the system.

The combination of these two effects result in the nucleation barrier, as shown below:\[\Delta G = -\frac{4\pi r^3}{3\Omega} \Delta \mu + 4\pi r^2 \gamma,\] (2-1)

where $\Omega$ is the volume of a molecule inside the crystal; $r$ is the radius of the nucleus; $\Delta \mu$ is the thermodynamic driving force, and $\gamma$ is the interfacial free energy per unit area between nucleus and solution. At first, $\Delta G$ increases with $r$ until it reaches a maximum for a value of $r$, called the critical radius $r_c$, and then decreases as $r$ tends to infinity. This means that a nucleus will be stable once it has grown up to the critical size $r_c$. The particular interest is that $\Delta G$ decreases with supersaturation and increases with the interfacial crystal/solution free energy. This means that a high supersaturation reduces the energy threshold to create a new phase and favors

![Fig. 2-1. Schematic illustration of the formation of nucleation barrier.](image-url)
nucleation. The presence of foreign particles reduces the free interfacial energy and increases the frequency of nucleation. Thus a lower supersaturation is required to nucleate when dealing with heterogeneous than homogeneous nucleation.

In the process of homogeneous nucleation, the nucleation barrier\(^2, 3, 67\) is then given for a spherical nucleus by

\[
\Delta G^*_{\text{hom, o}} = \frac{16\pi \gamma_{\text{sf}}^3 \Omega^2}{3[\Delta \mu]^2},
\]

and the critical size of the nuclei is

\[
r_c = \frac{2\gamma_{\text{sf}}}{\Delta \mu},
\]

\[
\Delta \mu = kT \ln(1 + \sigma),
\]

where \(\Delta G^*_{\text{hom, o}}\) is the nucleation barrier for homogeneous nucleation; \(k\) is the Boltzman constant, and \(T\) is the absolute temperature. In Eq. 2-4, \(\sigma\) is defined\(^{13, 65, 68, 69}\) as the supersaturation of solution, and for CaOx crystal, one has

\[
\ln(1 + \sigma) = \ln\left[a(Ca^{2+})a(C_2O_4^{2-}) / K_{\text{sp}}\right],
\]

where \(K_{\text{sp}}\) is the solubility product at a given temperature; \(a(Ca^{2+})\) is the activity of \(Ca^{2+}\), and \(a(C_2O_4^{2-})\) is the activity of \(C_2O_4^{2-}\).

### 2.1.2 The Introduction of a New Nucleation Theory

Since the association between the substrate and the biominerals is largely determined by heterogeneous nucleation\(^2, 3, 12, 14, 28, 47, 67, 70-72\), some nucleation theories examined the impact of the nucleation on the kinetics and the formation of the self-organized structure of biomineral aggregates. Here, a newly found nucleation theory is introduced. Considering the effect of the substrate on both the nucleation barrier and
the transport process, as illustrated in Fig. 2-2, the nucleation induction is given according to the model\textsuperscript{12, 14, 47, 67, 70-72} as

\[ J = \left( R^s \right)^2 N^o f''(m) \left[ f(m) \right]^{1/2} B \exp \left[ -\frac{\Delta G^*_{\text{homo}}}{kT} \right] f(m), \]  

(2-6)

with

\[ \Delta G^*_{\text{homo}} = \frac{16\pi\gamma^3_{ef}\Omega^2}{3kT \ln(1 + \sigma)}, \]  

(2-7)

and

\[ f''(m) = \frac{1}{2} (1 - m), \]  

(2-8)

\[ f(m) = \frac{1}{4} (2 - 3m + m^3), \]  

(2-9)

where \( R^s \) and \( N^o \) are the radius and the density of the substrates respectively; \( k \) is the Boltzmann constant; \( T \) is the absolute temperature; \( B \) is the kinetic constant; \( \Delta G^*_{\text{homo}} \) is the homogeneous nucleation barrier; \( \gamma_{ef} \) is the specific interfacial free energy between the crystals and the mother phase, and \( \Omega \) is the volume of the growth units.

In Eqs. 2-6 to 2-9, \( m \) depends on the interaction and (statistical) interfacial structural match between the crystalline phase and the foreign bodies, and is expressed as a function of the interfacial free energies between the different phases\textsuperscript{12-14, 47, 65, 67, 69-73}

\[ m = \left( \gamma_{sf} - \gamma_{sc} \right) / \gamma_{sf} \quad (-1 \leq m \leq 1). \]  

(2-10)

Fig. 2-2. Scheme of the process of nucleation at the surface of a foreign surface.
Here $\gamma_s$, $\gamma_c$ and $\gamma_f$ correspond to the interfacial tension between substrate and fluid, crystal and substrate, and crystal and fluid, respectively. In the presence of substrates the nucleation barrier assumes the form\textsuperscript{13, 65, 69, 73}

$$\Delta G^*_{\text{heter}} = \Delta G^*_{\text{homo}} f(m) \quad (0 \leq f \leq 1). \quad (2-11)$$

$f(m)$ is a factor describing the lowering of the nucleation barrier $\Delta G^*$ due to the occurrence of foreign bodies. If $f(m) \to 0$, then the $\Delta G^*_{\text{heter}}$ vanishes almost completely, this means the growing crystals are well oriented and ordered with respect to the structure of the substrate. While in the case of $f(m) \to 1$, the substrate exerts almost no influence on the nucleation, and the nucleation is controlled by the kinetics of homogeneous nucleation, which results in disordered\textsuperscript{13, 14, 47, 67-69, 73-75} nuclei. Obviously, this factor plays an important role in the determination of the heterogeneous nucleation barrier $\Delta G^*_{\text{heter}}$. The influence of foreign particles such as dust particles, proteins or even existing crystallites etc. on the nucleation barrier, and the association between the nucleating phase and the substrate can be fully characterized by this factor\textsuperscript{13, 14, 68, 69, 73}.

To study the nucleation kinetics, one of the most common ways is to measure the induction time $(t_s)$ of nucleation at different supersaturations. By definition\textsuperscript{12, 47, 67}, the nucleation rate $J$ can be expressed as

$$J \equiv 1/(t_s V), \quad (2-12)$$

where $V$ is the volume of the system. It follows then from Eq. 2-6 that

$$\ln t_s = \frac{\kappa f(m)}{[\ln(1 + \sigma)]} - \ln \left\{ \frac{V(R^*)^2 N^0 f^*(m) [f(m)]^{1/2}}{B} \right\}. \quad (2-13)$$
where \( \kappa = 16\pi r_{cf}^3 \Omega^3 / 3(kT)^3 \), which will remain constant under a given condition.

2.1.3 The Impact of Foreign Particles on the Heterogeneous Nucleation

Concerning the effect of a foreign body\(^{13, 14, 65, 68, 69, 73-75}\), most theories published so far mainly focus on the influence on the nucleation barrier\(^{2, 3, 12, 16, 20, 24}\). Actually, the occurrence of a foreign body will not only lower the nucleation barrier but also affect the transport of growth units to the surface of the crystalline clusters. As shown in Fig. 2-3, in the case of homogeneous nucleation, the growth units can be incorporated into the nucleus from all directions. However, nucleation on a foreign particle will cause a reduction in the “effective surface” of the nucleus, where the growth units are incorporated into the nucleus. This tends to slow down the nucleation kinetics, which cancels the effect of lowering the nucleation barrier. As a result, this will exert a direct impact on the formation of self-organized aggregates mediated by nucleation and can be described by the interfacial correlation factor \( f(m) \) and \( f''(m) \) in the previous discussion. These two contradictory effects play different roles in different regimes. At low supersaturations, where the nucleation barrier is very high, heterogeneous nucleation with an optimal structural match between the crystalline

![Fig. 2-3. Schematic illustration of the effect of foreign particle on the transport of structural units from the bulk to the nucleating sites. In comparison with homogeneous nucleation (A), the presence of the substrate blocks the collision of growth units onto the surface of the nucleus.](image)
phase and the substrate will be kinetically favored. In this case, the nucleation of crystalline materials will be best templated by substrates. However, at higher supersaturations, where the nucleation barrier becomes less important, instead of the nucleation barrier, it is the effective collisions, described by the factors $f(m)$ and $f''(m)$, that dominate in controlling the kinetics. Thus, nucleation on substrates with larger $f(m)$ and $f''(m)$ will be favored, and lead to a mismatch structure. As mentioned above, the templating of a substrate and the supersaturation-driven interfacial structure mismatch are two effects playing opposing roles in nucleation. Fabricating and engineering the complex structures of functional materials on the micro/nano scale can be achieved by carefully adjusting these two effects.

From Eq. 2-10, we know that $m$ is directly associated with $\gamma_{cs}$, which depends on the interaction and structural match between the nucleating phase and the substrate. For a given crystalline phase and a substrate, the optimal structural match at crystallographic orientation$^{65,75}$ corresponds to the strongest average interaction or the lowest interfacial energy difference. In general, the interfacial structure match between the crystalline phase and the substrate changes from a completely correlated and ordered state to a completely uncorrelated and disordered mismatch state as $m$ varies from 1 to -1. For instance, an excellent structural match $m\rightarrow1$ implies that $\Delta G_{\text{het}}^*$ vanishes almost completely. This occurs only when the growing crystals are well oriented and ordered with respect to the structure of the substrate. While in the case of $m\rightarrow-1$, the substrate exerts almost no influence on the nucleation, and the nucleation is controlled by the kinetics of homogeneous nucleation, which results in disordered nuclei. Due to the anisotropy of the crystalline phase, the available $m$
values should be a discrete set of values. Therefore, the structural match will deviate from the optimal structural match position to a secondary optimal structural match position. Consequently, \( m \) will shift from \( m=1 \) to a lower value. Since for the crystalline phase, \( m \) and \( f(m) \) take on only those values corresponding to some crystallographically preferred orientations, we expect to obtain a set of intersecting straight lines from the \( \ln t_s \) versus \( 1/[\ln(1+\sigma)]^2 \) plot\(^{65, 74, 75} \). These lines with different slopes \( k f(m) \) in different regimes indicate that nucleation is governed by a sequence of progressive heterogeneous processes. With increasing supersaturation, the interfacial correlation factor, \( f(m) \), subsequently increases, as \( k \) is constant for a given nucleation system. This unambiguously implies that an increase in supersaturation tends to drive the interfacial structure correlation between substrates and biominerals from a match state to a mismatch state.

### 2.2 Urinary Protein with the Calcium Oxalate Stone/Crystals

In the urine, the macromolecules have a controlling influence on the formation of urinary stone\(^2, 29, 36, 37, 76-79 \). Here, based on recent significant advances in the science and technology, some urinary proteins are presented with major impact on CaOx crystallization. Boyce and Garvey\(^76 \) pioneered the modern study of kidney stone protein. It is known that protein occupies much more space in CaOx stone, network throughout the entire structure of the stone and plays a key role in determining the architecture of calculi. The protein is commonly present as a series of concentric layers associated with radial striations that appear ordered, rather than random. While
the physical features of stone ultrastructure have been reasonably amenable to direct microscopic examination, its chemical composition has proved more difficult to explore. Despite the fact that stone matrix has been shown to contain an ever-increasing list of individual proteins, in most cases it is impossible to say with any certainty what kind of role they are playing. We will now present the details about several urinary proteins that have been subjected to rigorous study because they have shown significant influence on the crystallization of CaOx.

2.2.1 Tamm-Horsfall Glycoprotein

Tamm-Horsfall Glycoprotein (THG)\textsuperscript{76} is the most extensively investigated urinary protein in urolithiasis research, probably because it is the most abundant protein in human urine. THG is a renal protein of all placental invertebrates, localized to the luminal aspect of epithelial cells of the distal convoluted tubules and distributed throughout the epithelial cells of the thick ascending limb of the loops of Henle.

Despite its abundance in urine, THG is found only sparingly in stone matrix, and it is absent from CaOx crystals that precipitate from urine\textsuperscript{40}. Some research indicated that THG binds only weakly to CaOx crystals. Since it has been accepted that inhibitors act by binding to crystal surfaces, it was expected that THG was a poor inhibitor of CaOx crystallization. Unfortunately, the conclusion is not solid, because THG exhibits different properties depending upon the experimental conditions, and consequently, experimental findings are both confusing and contradictory. The protein has been reported to act as an inhibitor\textsuperscript{41, 44, 45} and also a promoter\textsuperscript{41, 43, 80}. The finding is further complicated by the fact that conflicting findings\textsuperscript{81} were obtained in the only studies in which the effect of THG was tested in undiluted urine: Hallson,
Rose and Sulaiman found that the THG enhanced the deposition of CaOx crystals from urine, which was concentrated by evaporation to high osmolalities. However, Ryall et al. and Grover et al. found that the protein was a potent inhibitor of CaOx crystal aggregation, although having no effect on CaOx crystal deposition. An explanation for these opposing findings is that while THG promotes CaOx crystal precipitation under conditions of high osmolality, where it also links CaOx crystals together to form large, loosely connected agglomerates, it is a very effective inhibitor of crystal aggregation at more usual urinary concentration. It is also proved that THG inhibits crystal aggregation by steric hindrance, not by binding to the crystal surfaces. The disagreement was also found in similar conflict relating to its urinary excretion. If indeed THG does play a directive role in stone formation, we might expect that its excretion would be different in stone formers and normal subjects, but it is not.

It would be fair to say that we have not reaped the bounty of study on THG. We know that the protein can act both as a promoter and an inhibitor of CaOx crystal processes in experimental crystallization systems, however we still cannot say with certainty whether it actually plays a key role in the formation of stones. Further studies are still required to elucidate its real contribution to urolithiasis, and its interaction, with its urinary companions.

2.2.2 Nephrocalcin

Nephrocalcin (NC) has also been the most widely studied protein reported in the stone literature. It was first described in 1978 and then for a number of years been deemed as a inhibitor of CaOx crystal growth. NC has been assumed a prominent
position in urolithiasis research, having been regarded as the principal inhibitor of CaOx crystallization in urine\textsuperscript{83}. It has been reported accounting for approximately 90% of urine’s total inhibitory effect on CaOx crystallization\textsuperscript{42,83}.

NC has been reported to occur in urine at concentrations\textsuperscript{83,84} ranging from 5mg/L to 16mg/L and to contain\textsuperscript{46,83,85} 2-3 residues of \(\gamma\)-carboxyglutamic acid (Gla) in its primary structure. The Gla component isolated from the urine of stone inhibit CaOx crystallization, however the NC isolated from the urine of stone formers was reportedly deficient in this amino acid\textsuperscript{86}, and the urine from these individuals had reduced inhibitory activity. A lack of Gla in NC isolated from kidney stones was suggested as the reason why the stones had formed\textsuperscript{86}.

However, a recent paper by Worcester\textsuperscript{87} et al. reassessed the inhibition effect of NC to the CaOx crystallization in urine to be no more than 16\%. Moreover, more researchers\textsuperscript{88,89} think that this inhibitor ability is shared with a number of other urinary proteins, such as uropontin, urinary prothrombin fragment 1 and uronic-acid-rich. The study of NC should be more carefully done to avoid the possibility of producing confusing discussions.

2.2.3 Uropontin (Osteopontin)

Uropontin (UP)\textsuperscript{76,89-91}, which reveals complete identity with the N-termini of Osteopontin (OP), has exhibited maximal inhibition of CaOx crystal growth in an inorganic metastable solution, however, its effect on crystal aggregation has not been determined\textsuperscript{92}. 
Osteopontin is an important protein in bone mineralization, where it is thought to anchor osteoblasts to bone \(^9\). Originally isolated from rat bone matrix as a 44 kDa phosphorylated protein, it is rich in serine, aspartic acid and glutamic acid-acidic amino acids commonly found in proteins involved in biomineralization\(^{93}\).

UP is abundantly founded in Calcium Oxalate monohydrate (COM)\(^{94}\) more than in Calcium Oxalate dihydrate (COD). In addition, its quantities in COM is substantially greater than that reported for NC\(^{86}\). UP is present in normal adult urine at a mean concentration of approximately \(6 \times 10^{-8}\) molar\(^{94}\). Some researches consider that it binds more avidly to the CaOx crystal surface than NC, and may consequently be a more potent inhibitor. However its inhibitory effect on CaOx crystallization has not been tested in urine\(^{94}\). Therefore, now, it is not possible to assess its potential effects on CaOx crystallization in vivo. Thus, more significant information must be obtained before it will be possible to claim with certainty that the presence of UP in urine is related specifically to its ability to inhibit CaOx crystallization, and thereby, stone pathogenesis.

**2.2.4 Urinary Prothrombin Fragment 1**

Urinary prothrombin fragment 1 (UPTF1)\(^{76}\) was isolated from CaOx crystals freshly precipitated from urine. Doyle et al.\(^{40}\) reasoned that the study of crystals enabled the study of urinary proteins, which was directly involved in the crucial crystals nucleation phase of stone formation, and thus eliminate any other macromolecule that might be introduced by cellular injury.
The presence of UPTF1 in CaOx stones\textsuperscript{95} is a consequence of direct inclusion into the crystalline architecture. Analysis of calcium phosphate crystal matrix reveals that UPTF1 is a major component, whereas in urate crystals it is only a very minor constituent\textsuperscript{95}. Limited data also demonstrated that the amount of UPTF1 in the kidneys of stone formers is significantly greater than those from healthy subjects\textsuperscript{96}. This is the finding, which has raised a number of research subjects that future research must address.

Until recently, evidence that UPTF1 inhibits CaOx crystallization was only indirect, UPTF1 is the most prominent protein in the organic matrix of CaOx crystals precipitated from fresh human urine\textsuperscript{94}. This, together with the observation that the organic matrix is the most potent macromolecular inhibitor of CaOx crystallization induced in human\textsuperscript{97}, led to the presumption that this inhibitory activity was attributable to UPTF1. This presumption was largely justified by the research\textsuperscript{98} that UPTF1 is now known to be potent inhibitor of CaOx crystal aggregation in undiluted urine. There seems little doubt that the potent inhibitory effect of UPTF1 on CaOx crystallization can be ascribed to the Gla domain of the peptide. Derived from its parent prothrombin, this region of the protein’s primary structure contains 10 Gla residues.

The study of UPTF1 is still in its early stages. Certainly, preliminary data would indicate that it possesses all the features expected of a significant macromolecular urinary inhibitor, including potent activity in undiluted urine. Nonetheless, the true role of UPTF1 must remain speculative until a cause and effect relationship between the protein and stone pathogenesis can be unequivocally demonstrated.
2.2.5 Uronic-Acid-Rich protein

Uronic-acid-rich (UAP) protein was first described in 1993 by Atmani et al\(^9\). Now, there is relatively little published information about UAP\(^7\), but it was stated undoubtedly very prominent in forthcoming stone literature. The inhibitory activity\(^9\) of UAP was determined in an inorganic CaOx crystallization system, where it strongly retarded CaOx crystal growth. And, it was also reported\(^9\) that this activity is reduced in stone formers compared with normal controls. The protein has also been isolated from rat urine\(^1\) and shown to possess very similar properties to the human urinary protein.

Despite having been the subject of investigation for some years, the true physiological function of UAP remains a mystery. It is possible that its clinical usefulness will also extend to the treatment of human kidney stones. Unfortunately, the effect of UAP on CaOx crystallization in human urine has not yet been determined\(^8\). It is clear, that there is an urgent need to clarify the role of UAP in stone formation.

2.2.6 The Questions Remaining

The study of stone proteins has come a long way in recent years\(^7\), but the knowledge we have gained so far has been offset to a large extent by conflicting findings, some of which have simply deepened the mystery of the role of proteins in stone formation. New technology has enabled us to identify all the involved proteins, but in every case, we cannot say with much certainty just why they are there – whether they are good, bad or indifferent. Much of the confusion and contradiction that abound in the literature concerning protein macromolecules can be ascribed directly to the habit, of drawing conclusions about macromolecules’ effects in stone formation from data
derived from aqueous inorganic or simple organic systems. Such systems do not reproduce the complex ionic milieu of urine and we cannot expect inhibitors to exhibit the same effects in splendid isolation at low ionic strength, as they would in the urinary soup. It is to be hoped that in the future, results derived from inorganic media will be regarded with an appropriate degree of caution and more information will be treated using crystallization systems based on urine. Of course, no experimental crystallization system will ever replace the surfaces, the fluid and concentration dynamics, the twists and turns of the environment of the human kidney.

As each new protein is added to the list of urine component, it is becoming increasingly apparent that there is no single ingredient that alone will carry the blame for the fact that some of us suffer from stones, or take the credit for the fact that the majority of us, happily, do not. Every protein is potentially an activity protagonist in stone pathogenesis until proven otherwise. Future research intent on identifying those macromolecules rightfully entitled to a place as participants in CaOx crystallization processes, should ensure that their effects are tested in urine, and not neglect the possible contribution of other urinary components, for this approach carries the promise of discovering their true role in stone pathogenesis.
CHAPTER THREE

Experimental Techniques and Materials

3.1 Applied techniques

To carry out the study on how the BSA influences the crystallization of CaOx, some techniques are utilized. Here, in this part, the following experimental setups are introduced: the Dynamic Light Scattering system (DLS), Scanning Electron Microscope (SEM), X-ray diffraction (XRD), Zetasizer and High Performance Particle Sizer (HPPS).

3.1.1 Dynamic Light Scattering

We noted that the Eq. 2-12, \( J = \frac{1}{t(V)} \), is potentially useful in studying the nucleation kinetics. From this equation, we know that the nucleation rate is inversely proportional to the induction time and the volume. Under a given condition, if \( V \) could be kept constant, we could find the direct correlation between the nucleation rate and the induction time. This can be an important step to obtain a set of consistent and reproducible data to study the nucleation kinetics. Therefore, it is necessary to find a reliable way to measure the induction time.
In our study, the Light Scattering system, Brookhaven BI-200SM dynamic Light Scattering (DLS) system, was employed to measure the nucleation induction time, as shown in Fig. 3-1. This device is armed with a He-Ne laser (632.8 nm) source, thus it can detect particles of size down to 2 nm, which allows an in situ measurement of the nucleation process and of the size increase of the nuclei.

Fig. 3-1. The picture of the Brookhaven BI-200SM Dynamic Light Scattering (DLS) system used in the study.

Fig. 3-2. Schematic illustration of the dynamic light scattering setup
Light Scattering occurs when polarizable particles in a sample are bathed in the oscillating electric field of a beam of light. The varying field induces oscillating dipoles in the particles and these radiate lights in all directions. The scattered intensity is proportional to the number and size of the particles. Light scattering has been employed in many areas of science to determine particle size, molecular weight, shape, diffusion coefficients etc. The schematic of the principle of the dynamic light scattering set up is illustrated in Fig. 3-2. In this study, by mixing the ingredients calcium chloride and sodium oxalate (CaCl$_2$ and NaOx) at time $t = 0$, the scattered light intensity is monitored to follow the nucleation and growth of CaOx crystals. The kinetics of nucleation can be examined because of the correlation between the nucleation induction time and supersaturation$^{101}$. During the crystallization process, what we normally measure is $t_{\text{measure}}$, which is defined as the mean time elapsing before appearance of an observable amount of the new phase (normally, this new phase is crystal). Actually, $t_{\text{measure}} = t_{\text{grow}} + t_{\text{nucl}}$ ($t_{\text{grow}}$ the time for the growth of crystals to be observed, $t_{\text{nucl}}$ is the induction time for nucleation). While, $t_{\text{nucl}} = t_{\text{nonst}} + t_s$ ($t_{\text{nonst}}$ is the transient period, it is the certain time required to establish nucleation from time zero to the steady state, and it is associated with nucleation of the nonstationary state; $t_s$ is the real induction time). It follows then that $t_{\text{measure}} = t_{\text{grow}} + t_{\text{nonst}} + t_s$. In this system, the laser light scattering method promises the detection of particles from several nanometer to several tens of nanometer, so the crystals with a sufficiently small size can be detected, then, we can have $t_{\text{grow}} << t_{\text{nucl}} (= t_{\text{nonst}} + t_s)$. We can even assume $t_{\text{grow}} \rightarrow 0$. In addition, $t_{\text{nonst}}$ is determined largely by the diffusivity of nucleation species. If the mother phase is not too viscous, the $t_{\text{nonst}}$ equal to a few microseconds. This implies that $t_{\text{nonst}} << t_s$. In conclusion, we can approximate that
\[ t_{\text{measure}} \equiv t_{\text{nucl}} \equiv t_s \]. In other words, this implies that under normal condition, the nucleation rate \( J \) is time-independent.

The DLS is controlled by a computer. The software screen is shown as Fig. 3-3. The left-up window is the control panel, which provides orders to run the DLS system, and from which some relative parameters can be set, changed and displayed. The right-down panel displays the result. From this display window, we can read out the induction time.

![Fig. 3-3. The controlling software of the Dynamic Light-Scattering system.](image)

### 3.1.2 Scanning Electron Microscope

The Scanning Electron Microscope (SEM) is extremely important in finding new applications in nanotechnology. With the advance of nano-fabrication techniques, the SEM technology has been developed to study the structures and topography in the
micro scale.

The SEM is a microscope that uses electrons rather than light to form an image, thus there are many advantages to use the SEM instead of a light microscope. The SEM has a large depth of field, which allows a large amount of the sample to be in focus at one time. The SEM can produce images of high resolution, which means that closely spaced features can be examined at a high magnification. As most SEMs only require the sample to be conductive, preparation of the samples is relatively easy. The combination of above mentioned merits make the SEM one of the most heavily used instruments in nano research areas today.

The SEM working principles is that: in vacuum situation, the scanning electron microscope generates an electrons beam. After being collimated by electromagnetic condenser lenses, and focused by an objective lens, the electrons beam scans across the surface of the sample by electromagnetic deflection coils. The primary imaging method is the collecting of secondary electrons that are released by the sample. The secondary electrons are detected by a scintillation material that produces flashes of light from the electrons. There is a photomultiplier tube used to detect and amplify the light flashes, whose illumination and shadowing show a quite natural looking surface topography. The resolutions of SEM are currently limited to around 25 Angstroms.

In this study, we used the JSM-6700F, which is a field emission scanning electron microscope (FESEM) incorporating a cold cathode field emission (FE) gun, ultra high vacuum, and sophisticated digital technologies for high resolution and high quality imaging of microstructures. Featuring a conical FE gun and a advanced objective lens, the system is capable of high resolution imaging as well as high quality real time
image display at all scan speeds, and capable of observation and recording of superior images.

The accessories include an auto fine coater (JEOL, JFC-1600). This coater, which consists of a main unit and a pump, is designed mainly to prepare specimens for SEM observation. As it is easy to set the chamber pressure and the sputtering current, the coater can coat biological and other nonconductive specimens with a thin layer of platinum to increase conductivity efficiently.

3.1.3 X-ray diffraction

X-ray diffraction (XRD) is the most important method for identifying the variety of crystalline mineral species. X-ray diffraction has been used in two main areas, for the “fingerprint” characterization of crystalline materials and the determination of their structure. The basic principle is that each crystalline solid has its unique characteristic X-ray powder pattern, which may be used as a "fingerprint" for its identification. X-ray diffraction is one of the most important characterization tools used in solid state chemistry and materials science. It is usually used in combination with a database of

![Bragg's law diagram](image_url)

*Fig. 3-4. Illustration of the Bragg’s law, the reflection of x-rays from two planes of atoms in a solid.*
known crystalline structures. The XRD method is non-destructive and requires only small amounts of material.

As illustrated in Fig. 3-4, the geometry generally used is the conventional 0-20 (or Bragg Brentano) geometry in which the angle of the diffracted beam equals the angle of incidence with respect to the sample surface. X-ray reflection takes place from lattice planes according to Bragg's Law: \( n\lambda = 2d\sin\theta \). Where \( d \) is the lattice spacing; \( \lambda \) is the wavelength of the X-rays; \( \theta \) is the glancing angle of reflection, and \( n \) is the order of the reflection, which can be any whole number.

In this study, we employed the Philips PW 1729 (Eindhoven, Netherlands) X-ray Its radiation is emitted by copper, whose characteristic wavelength for the K radiation is \( \lambda = 1.5418\text{Å} \). After the incident beam strikes a powder sample, the diffracted beam is detected by a moveable detector, which is connected to a chart recorder. In normal use, the counter is set to scan over a range of \( 2\theta \) values at a constant angular velocity. Routinely, a \( 2\theta \) range of 5 to 70 degrees is sufficient to cover the most useful part of the powder pattern, and scanning may cost about 30 minutes to obtain the trace.

3.1.4 Zetasizer

Almost all particulate or macroscopic materials in contact with a liquid can acquire electronic charge on their surfaces. A charged particle will attract ions of the opposite charge in the dispersant, forming a strongly bound layer close to its surface. Those ions further away from the core particle make up a diffuse layer, more loosely bound to the particle. Within this diffuse layer is a notional boundary, inside which the particle and its associated ions act as a single entity, diffusing through the dispersion
together. The plane of this boundary is known as the surface of hydrodynamic shear, or the slipping plane. The potential at this boundary is known as the zeta potential. Its magnitude is affected by both the nature of the particle surface, and the composition of the dispersant.

The zeta potential is an important parameter, which is related to the electrostatic repulsion between colloidal particles and thereby to the stability of the particles against aggregation. In addition, the zeta potential is a direct measure of the adsorption of charged molecules or ions on the surface of the particles, and a measure of the magnitude of the repulsion or attraction between particles. Its measurement brings detailed insight into the dispersion mechanism and is the key in electrostatic dispersion control. By measuring a concentration series, the point of zero charge can be determined.

The electrophoresis instrument utilized in the study is the Zetasizer 2000 of Malvern Instruments. It can measure the electrophoretic mobility of colloidal particles in an ionic solution and then calculates the zeta potential within certain assumptions.

### 3.1.5 High Performance Particle Sizer

Particle size measurement is an extremely important parameter across most branches of industry. A High Performance Particle Sizer (HPPS) is an instrument for measuring the size of molecules in solution or the size of particles in dispersion. The basic principle behind HPPS is Dynamic Light Scattering (DLS), also known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS). The DLS measures Brownian motion and relates it to the size of the particles. When a group of
particles is illuminated by light (usually laser), the light scattered by the particles will produce a speckle pattern consisting of areas of light and dark. Particles suspended in a liquid constantly move due to Brownian motion and hence the intensity of light in the speckle pattern keeps fluctuating. The DLS measures the rate of the intensity fluctuation using a digital correlator and uses it to calculate the size of the particles in the sample.

In this study, we employed the Malvern HPPS (High performance particle sizer), which can measure particles in the range $0.6\text{nm}$ to $6\mu\text{m}$. This setup can measure solution with the concentration ranging from 0.00001 vol\% (0.1ppm) to 20 vol\% with a temperature controlled range of $10 - 90^\circ\text{C}$, and only a very small sample of between $12\mu\text{l}$ to $3\text{ml}$ is required. Moreover, measurements can be made in conventional disposable plastic or glass cuvettes, eliminating the possibility of cross-contamination. The HPPS has the highest sensitivity of any dynamic light scattering (DLS) system available, and it uses a patented optical system called Non-Invasive Back-Scatter (NIBS).

### 3.2 Chemical Reagents

During this study, three reagent-grade chemicals: Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), Sodium Oxalate ($\text{Na}_2\text{C}_2\text{O}_4$) and Bovine Serum Albumin (BSA) were employed. The chemical reagents were weighed by electronic balance (Shimadzu, Aw220), with an accuracy of 1mg.
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, molecular weight of 147.02g/mol) was bought from Sigma Company with purity of 99%. It is odorless coarse white powder or a mixture of coarse white powder with medium sized granules. It is an irritant chemical and very toxic if inhaled. $CaCl_2 \cdot 2H_2O$ can freely dissolve in water with heat liberation, and forms a colorless solution. If $CaCl_2 \cdot 2H_2O$ is exposed in open containers, it will pick up moisture from the air and form granules, and finally go into solution. Since the calcium chloride dihydrate is hygroscopic, the measuring process was kept as short as possible. The purpose of minimizing the time that the reagent is exposed to the air is to reduce the mass changing resulted from the absorbed moisture from the air. Once diluted in water at the concentration of 1M, at $25^\circ C$, the pH of the aqueous solution ranges from 5.0 to 8.0.

The Sodium Oxalate ($Na_2C_2O_4$, molecular weight of 134.01g/mol) was bought from Merck Company, with the purity of 99.8%. It is harmful white powder, and contact with skin should be avoided. The solubility at $20^\circ C$ is 3.7g in 100g water, and the pH of the aqueous solution is around 7.0.

In the solution of $Na_2C_2O_4$, the $C_2O_4^{2-}$ undergoes two process:

$$C_2O_4^{2-} + 2H^+ \rightleftharpoons HC_2O_4^- + H_2C_2O_4.$$

Thus, the oxalate ion concentration must be estimated from the analytical value for total acid. This is done by calculating the ion fractions for the diprotic acid, oxalic acid. Since, $[Oxalate]_{total} = [H_2C_2O_4] + [HC_2O_4^-] + [C_2O_4^{2-}]$, the dissociation constants$^{27}$ of $H_2C_2O_4$ are $Pk_1 = 1.46$ and $Pk_2 = 4.40$. We have
In this study, the pH was adjusted to 6, then \( \left[ C_2O_4^{2-} \right]/\left[ \text{oxalatic} \right]_{\text{total}} \) equal 97.55%, which is extremely important in calculating the ion strength and consequently the supersaturation.

As the calcium chloride can cause irritation, especially to the eye and skin, and the sodium oxalate is toxic, to provide protection, goggle, gloves and the lab coat were necessary during the experiment.

The protein, BSA, was purchased from Sigma Company, with the purity of 98%. Normally, it should be stored at the temperature of 4°C in the refrigerator. It has a good binding capacity for water, \( Ca^{2+}, Na^{+}, K^{+} \), fatty acids, hormones, bilirubin and drugs. As the synthetic product of the liver and the main protein of plasma, albumin is a well-characterized protein with a molecular weight of \( 6.9 \times 10^4 \) and isoelectric point of about 6. Its main function is the regulation of the colloidal osmotic pressure of blood. Moreover, it will cause an allergic reaction in human.

The reaction equation of \( CaCl_2 \) and \( Na_2C_2O_4 \) can be expressed as:

\[
CaCl_2 + Na_2C_2O_4 + xH_2O = CaC_2O_4 \cdot xH_2O + 2NaCl \quad x = 1, 2, 3 \quad (3-3)
\]

Here, the \( CaC_2O_4 \cdot xH_2O \) denotes all of the three possible products, that is the three crystal phases. So, it could be \( CaC_2O_4 \cdot H_2O \) (COM, calcium oxalate monohydrate), \( CaC_2O_4 \cdot 2H_2O \) (COD, calcium oxalate dihydrate) and \( CaC_2O_4 \cdot 3H_2O \) (COT, calcium oxalate trihydrate). The products depend on the experimental conditions,
under which they are synthesized, such as the ion strength, temperature, the additives and so forth.

### 3.3 General Parameters of BSA

When the concentration is set to \(1 \text{mg/ml}\), at \(25^\circ C\), the measured size of BSA using HPPS is \(10.99 \pm 0.80 \text{nm}\). In this study, the membrane filter, whose pore size is \(0.2 \mu m\), and the filter paper (Grade 1, Whatman) with pore size of \(12-25 \mu m\) are employed to eliminate the effect of inevitable dust particles in the solution. The size measurement proved that the protein could freely pass through the filter and the filter paper and, in addition, the filtering process had not produced any negative effect on the study.

For a normal person, the pH of urine is around 6.0. Therefore, the zeta potential of

![Fig. 3-5. The Zeta Potential of the BSA. This shows that at conditions of the present study, the BSA almost has no charge.](image-url)
BSA at the pH is 6 is measured using the Zetasizer. According to the result (Fig. 3-5), we find that the zeta potential of BSA is almost zero, which means that in the presence study, the BSA is neutral. As pI is the pH value at which the molecule has no net charge, the pI of BSA is 6.0.
CHAPTER FOUR

CaOx Nucleation Kinetics

To make clear how the protein, BSA, influences the formation of CaOx crystal from the kinetic point, the experiment was carried out in two steps. The first is to study the formation of CaOx crystal without the protein (BSA). The second is to examine the influence of the protein. By comparing these results, the role played by the protein, albumin in the formation of CaOx crystal could be discovered.

4.1 X-ray Diffraction of CaOx Crystal

At first, the crystal phase must be verified to check whether the presence of BSA has changed the crystal phase. Therefore, the initial step is to carry out the X-ray Diffraction (XRD) patterns study.
4.1.1 Sample Preparation

To examine the crystal phase by the X-ray Diffraction, the first step is to prepare the crystal samples. The bulk solution of CaCl₂ and NaOx are prepared by dissolving the reagents with the DI water and then sonicating. The concentration of the two chemical reagents is fixed at, 0.7 mM, 1.0 mM, 1.5 mM, 2.0 mM, 3 mM and 5 mM separately. After this, the mentioned two kinds of solutions, of the same concentration are mixed slowly. During the mixing process, the solution changes from clear to opaque, and then small white particles are precipitated slowly. The mixture solution is filtered using a filter paper. Then, the filter paper, with the white particles adhered to it, is kept in an oven at 30°C for 20 hours. In this process, the filter paper loses moisture and becomes dry. From the dried filter paper, the crystal powder is carefully collected and transferred to the XRD device sample holder. After packing, the powder holder is loaded into the sample changer. Finally, the x-ray diffraction pattern is obtained.

The next step is to study the effect of BSA on the CaOx crystal phase. The method of sample preparation is almost the same as in the pervious step, except that while dissolving the CaCl₂ and NaOx, the albumin is added at different concentrations ranging from 40mg/L to 400mg/L.

The x-ray diffraction patterns obtained in the above two steps are compared with those of CaOx crystals listed by the Joint Committee on Powder Diffraction Standards (JCPDS) powder diffraction database.
4.1.2 The Influence of BSA on the CaOx Crystal Phase

The CaOx crystals, especially the Calcium Oxalate Monohydrate (COM) crystal structure is well known and thoroughly discussed\textsuperscript{18, 102}. According to the JCPDS database, calcium oxalate monohydrate\textsuperscript{8, 18, 31, 33, 52} has monoclinic symmetry and belongs to the $P2_1/c$ space group with parameters $a=6.29$, $b=14583$, $c=10.116$, $\beta=109.46$. While the Calcium Oxalate Dihydrate (COD)\textsuperscript{31, 33} has tetragonal symmetry and belongs to the $I4/m$ space group with parameters $a=12.35$, $c=7.363$. The Calcium Oxalate Trihydrate (COT)\textsuperscript{103} crystal has triclinic symmetry, with the parameters as $a=6.076$, $b=7.174$, $c=8.467$, $\alpha=76.65$, $\beta=70.85$, $\gamma=70.91$. Among them, the COM is thermodynamically more stable than COD, and COD is more stable than COT.

The XRD pattern of CaOx crystals obtained from the solution without BSA is shown in Fig. 4-1. By comparing with those of CaOx crystals listed by the Joint committee on Powder Diffraction Standards power diffraction data, the result shows that the crystal is COM. However, with the presence of BSA, the result is different. The XRD pattern of CaOx crystals obtained from the solution in the presence of BSA is shown in the Fig. 4-2. This figure shows that the sample crystals are a mixture of COD and COM. From the JCPDS data, we know that the crystal faces with open circle indicate the presence of COM, and the asterisks indicate the presence of COD crystal. Moreover, the intensity indicates that, in the sample, the majority is COM, while only a small portion is the COD. These experimental results indicate that the presence of BSA favors the formation of COD crystal.
CHAPTER FOUR  
CaOx Nucleation Kinetics

Fig. 4-1. The XRD pattern of CaOx crystals obtained from the solution without BSA. By comparing with those of calcium oxalate crystals listed by the Joint committee on Powder Diffraction Standards powder diffraction data, the result confirmed that the crystal is COM.

Fig. 4-2. XRD pattern of CaOx Crystals obtained from the solution with the BSA. The crystal faces with open circle indicate the presence of COM crystal. The asterisks indicate the presence of COD crystal.
4.1.3 The Medical Effect of COD and COM

The analysis of CaOx crystals in urine shows that the dihydrate form is present in healthy subjects and stone formers, whereas the monohydrate, which is thermodynamically more stable and constitutes the core of most urinary stones, is present in stone formers only. So promotion by albumin of CaOx crystallization with specific formation of dihydrate form might be protective\textsuperscript{32, 51}. In addition, it is known that COM is prevalent in kidney stones\textsuperscript{32, 51}, and COD is more commonly excreted in urine. Consequently, it has been proposed that preferential formation in vivo of COD, rather than COM, protects against stone formation because they are less likely to adhere to renal tubular cells. It is also evident that COM crystals are more injurious to cell membranes than are COD crystals, and the binding of COM and COD crystals to cultured renal cells is with different face-selective affinities. Thus, the COM more easily adheres to the well of the kidney and/or renal tubule than COD. Therefore, the difference between the mono- and dihydrate forms of calcium oxalate might determine whether crystals are expelled harmlessly in the urine or are retained within the kidney and induce the formation of calculi. The above mentioned process and

Fig. 4-3. Scheme showing of a renal tubule, in which supersaturated urine with CaOx is flowing. The arrow indicates the flow direction of the urine. In the urine, after the nucleation and growth of CaOx, most of the COM is bonded to the renal tubule, while most of the COD is propelled out.
effect is illustrated in the Fig. 4-3. In the urine, after the nucleation and growth of CaOx, most of the COM is bonded to the renal tubule, while most of the COD is propelled out.

4.2 CaOx Nucleation Kinetics Study

4.2.1 Sample Preparation

Sample preparation consists of three parts: glassware cleaning, solution preparation, and solution purification. The first and the third steps share the same major feature, to alleviate the possibility that dust influences the experiment data. This is because: On the one hand, in the light scattering measurements, dust is a major problem. Dust is a general name given to any undesirable, large scatter that contributes to the signal. As in our experiment, the light scattering system cannot distinguish the dust from the newly born crystals. This can produce puzzling data. Most often dusty samples limit the reproducibility of the results. Unfortunately, dust can also yield systematic errors. Dust is much more of a problem in polar liquids, especially in water. On the other hand, the dust can also affect the nucleation process of the CaOx crystal. It will not only work as the substrate that will promote the nucleation by lowering the nucleation energy barrier, but also work as the additive that could adhere to the nucleation and growth kinks sites, thus slow down the nucleation and growth rate. Therefore, the dust must be removed as much as possible.

To remove the dust, the glassware and reagent solutions were treated separately in two different ways. For the glassware, all the beakers, glass cells, flask and other
glassware were washed carefully first by chromium acid, then by DI water. This process was repeated twice, and finally the glassware was cleaned by acetone. The purpose of this whole process was to make sure that all the unwanted dust and organic contaminant could be washed away.

Stock solutions were prepared by dissolving reagent-grade chemicals in deionized water and sonicated. Dozens of samples with concentrations ranging from 3.6mM to 5.2mM of were prepared by gradually diluting the stock solutions. Then, the filter was utilized to remove the dust and other impurity particles in the solution. Multiple passes were necessary because filters cannot trap all the dust during the first passage.

Both kinds of solutions (calcium chloride and sodium oxalate), with the same concentration, were injected into the cells of light scattering system to examine the nucleation kinetics of CaOx formation from the reactants. The induction time $t_s$ was measured by monitoring the photon count rate recorded by the DLS equipment against time. Subsequently, to simulate the body fluid, the salt sodium chloride was added at the concentration of 140mM. Separately, the BSA was also added to the solutions with the concentration of 0.5mg/L and 1mg/L, to study its effect on the CaOx nucleation.

4.2.2 The Effect of Supersaturation and Ion Activity on Nucleation Kinetics

Based on Eq. 2-13, and related discussion, we know that the plot of $\ln(t_s)$ versus $1/[\ln(1 + \sigma)]^2$ can be made. Thus, for a given system ($\kappa$, $B = $ constant), we can determine the $\kappa f(m)$, which is the major factor in the study of the nucleation
kinetics. Therefore, we can analyze the nucleation kinetics under various effects, such as the supersaturation and the substrate.

To examine the influence of the supersaturation on the nucleation kinetics, for CaOx crystal, the first step is to calculate the ion activity and supersaturation. The supersaturation of a system may be expressed in a number of different ways, and considerable confusion could be caused if the basic units of concentration are not clearly defined. The first statistical theory of electrolyte solutions, developed by Debye and Hückel in 1923, enabled the successful interpretation of the behavior of ions in dilute solution.

To utilize the Eq. 2-5, the ion activity should be calculated first. According to Debye and Hückel, the effective concentration of an ion in solution (expressed as its chemical activity) differs from its actual concentration by a factor dependent on the electrical field strength (as defined by the ionic strength) of the solution. In simple terms, this correction allows for the nonspecific effects of other ions in the solution on the true chemical activity of the ion under consideration. To obtain the activities of the ions in the solution, we first calculate the ionic strength, given as

\[ I = \frac{1}{2} \sum c_i z_i^2. \]  

(4-1)

Where \( c_i \) represents the concentration of the \( i^{th} \) ion and \( z_i \) the ion charge. According to the Extended Debye-Huckel theory, the activity coefficient \( \gamma \) can be calculated by not only taking the ion size into account, but also the temperature and the solvent effect, while resulting improved accuracy of the prediction. Therefore, the following expression of the activity is applicable for solutions having much higher ionic strengths, especially for the multi-ionic systems:
\[
\log \gamma = \frac{-0.51z^2\sqrt{\Gamma}}{1 + \left(\frac{\alpha}{305}\right)}
\]  

(4-2)

where \(\alpha\) is the hydrated ionic radius. In conclusion, the activity of the ions can be obtained using the equation

\[
a = \gamma \cdot c.
\]  

(4 - 3)

Based on above discussion, equations and experiment data, the \(\ln(t_s)\) versus \(1/\ln(1 + \sigma)\) plot for the CaOx homogeneous nucleation was obtained, as presented in Fig. 4-4 (A). According to Eq. 2-13, for a given system \((\kappa, B = \text{constant})\), we can analyze the effect of the supersaturation in terms of the variation of the slope, \(\kappa f(m)\), of these plots. As shown in Fig. 4-4 (A), the curve can principally be fitted by two intersecting straight lines with different slopes. As illustrated in Fig. 4-4 (B), with the supersaturation increases, \(f(m)\) increases abruptly, at the point where the supersaturation equals 780.15. This indicates that the nucleation is controlled by two discrete values of \(f(m)\) within the corresponding regions. From Fig. 4-4 (B), we know that in region I, where the supersaturation is lower than 780.15, the \(\kappa f(m)\) equals 1016.5, while in region II, where the supersaturation is higher than 780.15, the slope equals 2579.1. These lines with different slopes \(\kappa f(m)\) in different regimes indicate that nucleation is governed by a sequence of progressive homogeneous processes.

The \(\ln(t_s)\) versus \(1/\ln(1 + \sigma)\) plot for the CaOx crystal nucleation under the effect of NaCl is presented in Fig. 4-5 (A). The curve can also be fitted by two intersecting straight lines with different slopes. Since \(\kappa\) is constant, as shown in Fig.
Fig. 4-4 (B). Plot of $f(m)$ for CaOx homogeneous nucleation. With the increase of supersaturation, the interfacial correlation factor $f(m)$ will increase abruptly at a certain supersaturation.
4-5 (B), when the supersaturation increases, $f(m)'$ increases abruptly at the point, where the supersaturation is 2273.94. The slopes are 8164.7 in region II', where the supersaturation is higher than 2273.94, and 1778.6 in region I', where the supersaturation is lower than 2273.94. This indicates that the nucleation is also controlled by two discrete values of $f(m)'$ within the corresponding regions. These lines with different slopes $\kappa f(m)'$ in different regimes indicate that nucleation is still governed by a sequence of progressive homogeneous processes with the addition of NaCl, which provides the buffering effect.

By comparing Figs. 4-4(A) and 4-5(A), we find that with the addition of NaCl, at the same induction time, the concentration of CaCl$_2$ and Na$_2$C$_2$O$_4$ increase dramatically. This indicates that in the presence of NaCl, the nucleation induction time for CaOx crystal increases, thus the nucleation process is prolonged. However, the activities of Ca$^{2+}$ and C$_2$O$_4^{2-}$ will not change much due to the buffering effect of additive ions, which can influence the activity coefficients by changing the ionic strength. This implies that the presence of NaCl inhibits the nucleation of CaOx crystal; moreover, the accumulation of supersaturation of CaOx crystal for nucleation in a biological system could be buffered, due to the high concentration of other ions. It follows that the effect of the supersaturation-driven interfacial mismatch can be suppressed to some extent by increasing the ion strength. Moreover, in the kidney, the passage time of urine through the narrowest part of the urinary tract, the nephron, is approximately three minutes. Therefore, the influence of NaCl to increase the induction time may allow the crystals formed to remain small enough during that time to be excreted freely$^{28}$. These can be parts of the reasons that though the urine is supersaturated with CaOx crystal, many of us still do not have the kidney stone.
Fig. 4-5 (A). Schematic plot of $\ln t_s \sim 1/[(\ln(1+\sigma))^2]$ for CaOx homogeneous nucleation under the buffer effect of NaCl. Two fitted lines with different slopes intersect each other, dividing the space into two regimes.

Fig. 4-5 (B). Plot of $\kappa f(m) \sim \sigma$ for CaOx nucleation with the effect of NaCl. With the increase of supersaturation, the interfacial correlation factor $f(m)'$ will increase abruptly at a certain supersaturation.
4.2.3 The Effect of BSA on Nucleation Kinetics

In order to examine the influence of the BSA on the nucleation kinetics, and the subsequent structure correlation effect on the CaOx crystallites, the $\ln(t_s)$ versus $1/[\ln(1 + \sigma)]^2$ plots were constructed for CaOx crystal when BSA was fixed at a series of concentrations, as presented in Fig. 4-6 (A). According to Eq. 2-13, for a given system ($\kappa; B = \text{constant}$), we can analyze the change of the structure correlation between the substrate and the crystalline phase in terms of the variation of the slope of these plots. As shown in Fig. 4-6 (A), each of these depicted curves can be fitted by two intersecting straight lines with different slopes. This indicates that the nucleation is controlled by two discrete values of $f(m)$ within the corresponding supersaturation regions. As the supersaturation increases, $f(m)$ increases sharply. This implies that the structural match between the substrate and the nucleating crystallites deteriorates as the supersaturation increases. Moreover, with the presence of the BSA and the increase of BSA concentration, the $\ln(t_s)$ versus $1/[\ln(1 + \sigma)]^2$ plots shift left, which indicates that the induction time increases, thus the CaOx nucleation process is prolonged. As the urinary stone formation is a time-restricted process, increasing the induction time may allow the crystals to remain small enough to be excreted out freely.28

The supersaturation at which $f(m)$ changes is defined as $\sigma^*$: A larger $\sigma^*$ implies that good crystalline alignment will be achieved in a wider range of supersaturations.65 In Fig. 4-6 (A) and Fig. 4-6 (B), $\sigma^*$ for curve 1, curve 2 and curve 3 is $7.01 \times 10^2$, $8.6 \times 10^2$ and $9.4 \times 10^2$, respectively. The fact that $\sigma_1^* < \sigma_2^* < \sigma_3^*$ shows that the $\sigma^*$
Fig. 4-6 (A). Plot of $\ln t_s (\text{sec}) \sim \frac{1}{[\ln(1 + \sigma)]^2}$ for calcium oxalate crystal nucleation under different conditions. Curve 1, no additive; Curve 2, with BSA at 0.5mg/L; Curve 3, with BSA at 1mg/L.

Fig. 4-6 (B). Plot of $\kappa f(m) \sim \sigma$ for CaOx nucleation, with the influence of BSA at different concentration. Curve 1, no additive; Curve 2, with BSA at 0.5mg/L; Curve 3, with BSA at 1mg/L.
exhibits a monotonic increase with BSA concentration. This implies that the presence of BSA plays a crucial role in suppressing the supersaturation-driven structure mismatch effect occurring in the crystalline assembly over a wider range of supersaturations. Since BSA can prolong the range of supersaturation for good structural match, we deduce from the above nucleation kinetics discussion, that a better CaOx crystallization assembly could be obtained in the presence of BSA.

### 4.2.4 How Can the BSA Affect the CaOx Nucleation Process

In the following, we will discuss how does BSA prolong the nucleation process of COM. First, let us review the general nucleation process: the growth units (molecules or ions) in the solution may, on collision, join into groups of two or more particles to form embryos. To reach a thermodynamically stable state, the embryos must overcome the nucleation energy barrier, thus attaining the “growth” of embryos. During the “growth” of an embryo, growth units should be transported from the bulk solution to the surface of the embryo and become incorporated into the kink sites. However, when foreign bodies (impurities and/or protein molecules) are present, they become incorporated on the nucleus surface. This not only suppresses the nucleation barrier, but also affects the transport of growth units to the surface of the crystalline clusters. The foreign body causes a reduction in the “effective surface” of the nucleus, which would normally be available for the growth units to incorporate themselves into the nucleus. This tends to slow down the nucleation kinetics, and increase the nucleation induction time.

When solute molecules (growth units) are transported to the kink sites at the surface of an embryo [Fig. 4-7 (A), and Fig. 4-7 (B)], the probability for these molecules to
be incorporated to the embryo depends on various factors: the complexity of the molecules, the structure of solid-fluid interfaces, the adsorption of solvent or impurity molecules, and so forth. It has become a common practice to control nucleation by introducing additives. The effect of additives or impurities on the nucleation process, in terms of the structure of interfaces, is discussed here.

To exert the effect of impurities or additives on nucleation, their molecules should adsorb onto the surface of embryo and/or the surface of substrate. Before growth units can be incorporated into the crystalline phase at the kink sites, the adsorbed impurities and solvent molecules should be removed (desorbed) first, as shown in Fig. 4-7 (A). Associated with this process, an energy barrier, so-called desolvation energy barrier $\Delta G_{kink}^+$ needs to be overcome. The kinetics of kink integration can be described by the kink kinetics coefficient $\beta_{kink}$, which by definition is given by:

$$\beta_{kink} \approx v \exp(-\Delta G^+/kT),$$

where $v$ is the frequency of vibration, and $\Delta G^+$ denotes the free energy barrier of kink integration. It follows from recent theoretic analyses that $\Delta G^+$ should include the desolvation energy barrier $\Delta G_{kink}^+$, the energy required to remove solvent or impurity molecules adsorbed at the kink sites, as shown in Fig. 4-7 (A).

Obviously, the adsorption of additives on the surface of growing embryos will change $\gamma_{cf}$ to a small extent. However, as shown in Fig. 4-7 (B), to remove (desorbed) the additives (in this study, it is BSA) on the crystal surface or the kink sites, the desolvation energy barrier, $(\Delta G_{kink}^+)'_{add}$ should be overcome. As illustrated in Fig. 4-8,

$$\Delta(\Delta G_{kink}^+)'_{add} = (\Delta G_{kink}^+)'_{add} - \Delta G_{kink}^+. \quad (4-5)$$
Fig. 4-7 (A). In the process of CaOx nucleation, water molecules enter kink sites on the embryo surface and kink site. They suppress the approach of growth units to the embryo.

Fig. 4-7 (B). Illustration of adsorption of BSA molecules at the kink site and embryo surface. In the process of nucleation, the adsorption of additives at the kink sites suppresses the approach of growth units to the embryo.
In conclusion, the dominant effect of the additives is the enhancement of the desolvation energy barrier, which slows down the kink kinetics.

Referring to others’ research conclusion\textsuperscript{72, 74}, in the plot of $\ln t_s \approx 1/[\ln(1 + \sigma)]^2$, if $\Delta y$ is the vertical shift of the straight lines, we have:

$$\Delta y = \Delta(\Delta G_{\text{kink}}^+)_{\text{add}} / kT,$$

(4-6)

where $\kappa = 16\pi\gamma c_0^3 \Omega^2 / 3(kT)^3$, as illustrated in Eq. 2-13. Therefore, in the plot of $\ln t_s \approx 1/[\ln(1 + \sigma)]^2$, once the vertical distance of these straight lines along the $y$ axis is calculated, the change in the desolvation energy can be identified. Consequently, the integration of growth units into crystal nuclei at the kink site will be significantly slowed down or even terminated due to the increase of the kink kinetics energy barrier\textsuperscript{49, 65, 75}.

In our experiment, the presence of BSA inhibits crystal nucleation by absorbing at the kink sites. Thus, it increases the kink kinetics energy barrier and the induction time.

![Diagram](image)

**Fig. 4-8.** In the process of nucleation, the adsorption of additives at the kink site enhances the kink kinetics barrier by $\Delta(\Delta G_\text{kink}^+)_{\text{add}} = (\Delta G_\text{kink}^+)_{\text{add}} - \Delta G_\text{kink}^+$.\]
According to above discussion, in Fig. 4-6 (A), the vertical distance of the straight lines along the $y$ axis equals $\Delta (\Delta G^*_{kink})_{add} / kT$, from which we can calculate the change of the kink kinetics energy barrier $\Delta (\Delta G^*_{kink})_{add}$. We calculated this quantity for $[Ca^{2+}] = [C_2O_4^{2-}] = 2.1mM$, in the presence of BSA at the concentration of 0.5mg/L and 1mg/L, and found that the energy barrier increase with reference to the case of homogeneous nucleation (where BSA is absent) is $0.62kT$ and $1.07kT$, respectively. When $[Ca^{2+}] = [C_2O_4^{2-}] = 2.35mM$, the energy barrier increase from homogeneous nucleation to nucleation in the presence of BSA of 0.5mg/L is $1.18kT$. However, when $[Ca^{2+}] = [C_2O_4^{2-}] = 2.45mM$, the energy barrier increase with the increase in the BSA concentration from 0.5mg/L to 1mg/L is $1.18kT$. 
CHAPTER FIVE

CaOx Morphology Study

From the previous chapter, we know that the BSA not only influences the CaOx nucleation kinetics, but also affects the crystal morphology. The experimental results of the previous chapter lead to the conclusion that the presence of BSA inhibits the CaOx crystallization and promotes a better crystal assembly in a wider range of supersaturation. However, as these conclusions are deduced only from the kinetics part, they should be treated with caution. Therefore, in this chapter, the CaOx crystal morphology is investigated to confirm the previous conclusions from kinetics study, and to elucidate the role the albumin plays in the CaOx crystallization.

5.1 Sample Preparation

To study the morphology change of the CaOx crystal, proper preparation of the samples is one of the most crucial steps. Only well-prepared samples can provide reproducible and reliable information, as any mistake would lead to unrepeatable results and wrong conclusion. In this section, the method to prepare the samples is introduced step by step in detail.
To examine the samples by SEM, cover glass was employed as the substrate to transfer the samples. At the beginning, the cover glass was cut into small square pieces of side length about 4mm, then was washed carefully using chromium acid, acetone and DI water several times to clean the contaminants adhered on the surface.

Second: to obtain the crystals, the solutions of CaCl$_2$ and NaOx were prepared separately by dissolving the reagents in deionized water to get the solution with the concentrations of 0.15mM, 0.2mM, 0.35mM, and 0.75mM. The solutions were sonicated for 20 minutes to increase the speed of dissolving. Then these two kinds of solutions, of the same concentration, were mixed slowly. During the mixing process, the solution changed from clear to opaque, and small, white powder slowly precipitated at the bottom.

Third: the small glasses prepared in the first step were dropped into the solution produced at the second step. These glasses settled at the bottom of the solution, and some crystals could adhere to the glass surface. Then, the sample solution was sealed by laboratory films and transferred to an oven, which was kept at a constant temperature of 37°C.

Fourth: 20 hours later, the cover glass was removed from the solution and transferred into acetone using tweezers. In fact, when the samples were taken out of the solution, inevitably there would be some solution adhered to the glass surface. The solution would start evaporating once the cover glass was exposed to air. During the vaporization process, the concentration of the adhered solution would increase, and new crystal phase could form. This would cause an ambiguity that the crystal samples on the small glass were not those formed in the mixed solution, but those formed after
the glass had been taken out of the solution. To resolve this problem, the acetone was used to clean the solution adhered on the glass surface. As acetone can mix freely with water, the unwanted solution would dissolve into the acetone, and leave the glass surface. In this way, we guaranteed that the crystals examined corresponded to those formed at the required supersaturation.

Fifth: the samples washed by acetone in the previous step were dried in the air. As the evaporation process of acetone is very fast, several minutes later, the acetone adhered on the glass disappeared leaving the glass dry.

Sixth: the samples were then transferred to the SEM accessory, an auto fine coater. At the environment of nitrogen gas, with the pressure lower than $5 \text{ Pa}$, a thin layer of platinum was coated on the sample surface. The purpose of this step is to increase the conductivity of the samples, thus making it observable by the SEM.

In the last step, the samples were put on the sample stage of SEM and transferred to the sample chamber. Once the pressure of the chamber was lowered down to $9.63 \times 10^{-5} \text{ Pa}$, the observation started.

In the study of examining the influence of ion strength on the crystal morphology, the sample preparation steps were the same as the mentioned above, except that sodium chloride (NaCl) was added to the mixture. In order to simulate the human body fluid, the sodium chloride concentration in the mixture was fixed at 140mM.

As this study investigates the effect of the protein, BSA, on the crystal morphology, the above sample preparation procedure was repeated by adding the albumin to the
bulk solution at beginning. The concentration of protein was controlled in a large range from 10mg/L to 400mg/L, regardless of the supersaturation.

The most important consideration in the method of preparation of the samples is that there are two consequences that could influence the crystallization process. The first is that, during the experiment, as the two bulk solutions are mixed together to produce crystal samples, inevitably turbulence is triggered, which increases the collision chance of ions. So the crystal nucleation and growth can be promoted. Moreover, the wall of the container and the inevitable existence of dust would play the role of substrate, whose templating effect would definitely lower the nucleation barrier, thus prompting the crystallization. Both of these consequences promote the crystallization process. This experimental method has these two unavoidable effects, so the results should be carefully discussed in order to avoid the possibility of reaching misleading conclusions.

5.2 CaOx Morphology Study

First, we found that the addition of sodium chloride to the solution did not produce much noticeable effect on the morphology change of the crystal. As we have discussed in the kinetics part, the addition of sodium chloride only enhances the ionic strength, and provides the buffer effect for nucleation. Moreover, based on the new nucleation theory, these changes only influence the nucleation barrier, not the crystal morphology. This conclusion has been confirmed by the crystal morphology study.
When the concentrations of $Ca^{2+}$ and $C_2O_4^{2-}$ were set to 0.15$\text{mM}$, no matter whether the BSA was present or not, the chance of detecting a crystal using SEM was very small. In most cases, we could not observe anything, except at very few times, we could catch a few small crystals, which were too small to be studied.

This result can be explained by the following two reasons. According to Eq. 2-13 and Eq. 4-3, when $[Ca^{2+}]=[C_2O_4^{2-}]=0.15\text{mM}$, the supersaturation is 7.3, which is only about $0.7 \sim 1.2\%$ of those investigated in the nucleation kinetics part. The supersaturation is so low that it is difficult to form enough large crystals to be observed by the SEM setup. However, as the experiment has been carried out by mixing the two bulk solutions, which inevitably caused turbulence resulting in an increase in the chance of ion collisions, the crystal nucleation and growth is promoted. What is more, the walls of the container and dust would play the role of substrate, whose templating effect definitely lowers the nucleation barrier, thus prompting crystallization. However, the correlated effect of the above-mentioned reasons leads to the result that only a few crystals have been observed.

When the concentrations of $Ca^{2+}$ and $C_2O_4^{2-}$ are set to 0.2$\text{mM}$, the corresponding supersaturation is 13.2. Under this condition, excellent SEM images of the crystals were obtained, as shown in Fig. 5-1. This picture shows the SEM image of a twined COM obtained from the solution without the presence of BSA. However, once the BSA is added with the concentration higher than 20$\text{mg/L}$, we hardly observe any crystals, even though other conditions, such as supersaturation, pH and temperature, were kept unchanged. If the concentration of BSA is lower than 20mg/L, the effect of BSA is not very obvious on the crystal morphology.
This result is in accordance with the results obtained from the kinetics part and the related discussions. Based on the new nucleation theory and the kinetics results, we had arrived at the conclusion that the BSA can inhibit the nucleation of CaOx crystal. This conclusion was deduced merely from the fact that the presence of BSA increased the induction time, and increased the supersaturation range to obtain better crystal assembly. These conclusions are concretely confirmed by the SEM pictures. When the supersaturation equals 13.2, in the presence of BSA, we have observed hardly any crystals; however, twined crystals have been observed if the BSA is absent. (As shown in Fig. 5-1)

According to the newly found nucleation theory, the BSA in the solution plays the role of template. On one hand, it lowers the nucleation barrier, thus promoting the

Fig. 5-1. The SEM picture of COM twined crystal obtained from a solution at low concentration (\([Ca^{2+}]=[C_{2}O_{4}^{2-}]=0.2mM\)) without additives. Scale bar, 5\(\mu\)m
nucleation. On the other hand, the BSA can be incorporated into the embryo and/or crystal surface and kink sites. This effect reduces the effective nucleation surface and lowers the number of available kink sites for the incorporation of growth units. Thus the energy barrier to be overcome for crystal nucleation and/or growth increases. In this part of experiment, the combination of these two conflicting roles of BSA leads to the pictures we have shown.

Fig. 5-2 is the SEM micrograph of randomly oriented COM crystals obtained from a solution, without the BSA, at high concentration \([Ca^{2+}] = [C_2O_4^{2-}] = 0.35mM\), the supersaturation being 38.06. This picture shows COM crystal with the disordered and highly aggregated morphology. However, in the presence of BSA, keeping other conditions unchanged, an ordered COM assembly and the co-occurrence of COD and COM can be obtained, as shown in Fig. 5-3. For a clear view of the COD, a single COD crystal is also shown in Fig. 5-4.

It is clear that the disordered crystal in Fig. 5-2 is caused by the supersaturation driven interfacial mismatch effect. During the kinetics study, we have deduced that the supersaturation driven interfacial mismatch effect could be alleviated by the BSA. This deduction has been confirmed by Fig. 5-3, which shows better ordered COM crystal than that in Fig. 5-2. This confirms that the synergy match effect can be achieved between the BSA and the COM, thus producing better crystal morphology and alleviating the interfacial mismatch effect. In the crystallization process of CaOx, the BSA performs templating effect, which is normally believed to improve the crystal morphology. The templating effect has two consequences: first, it lowers the nucleation energy barrier, thus promoting the nucleation; second, the BSA can be incorporated to the kink sites and/or the embryo surface, which will slow down
Fig. 5-2. SEM micrograph showing COM crystallites obtained from a solution at high concentration ([Ca$^{2+}$] = [C$_2$O$_4^{2-}$] = 0.35mM) without additives. Scale bar, 5μm

Fig. 5-3. SEM micrograph showing COM and COD crystallites obtained from a solution at high concentration ([Ca$^{2+}$] = [C$_2$O$_4^{2-}$] = 0.35mM) with BSA used as an additive. Due to the template effect of the biosubstrate, the crystallites show good structural synergy. Scale bar, 5μm
the nucleation process and/or crystal growth process. At the same time, the supersaturation always produces the supersaturation driven interfacial mismatch effect. This effect is more prevailing at higher supersaturations. Here, the two effects work against each other. Without the BSA, the supersaturation interfacial mismatch effect is the controlling factor, so in Fig. 5-2, the crystal has a disordered morphology. However, in the presence of BSA, the ordered COM morphology has been achieved by the templating effect, as seen in Fig. 5-3.

In addition, the presence of BSA has also favored the formation of COD, as shown in Fig. 5-3 and Fig. 5-4. This is in agreement with the XRD experiments and discussion too. According to others’ research work\textsuperscript{18, 102}, we know that the COD is less harmful to the kidney cell and urinary tubes than COM, and COD has a poor adherence ability than the COM. So the presence of BSA can be deemed as a reason that could trigger

![Fig. 5-4. SEM micrograph of a COD crystal, obtained from a solution at high concentration ([Ca\textsuperscript{2+}] = [C\textsubscript{2}O\textsubscript{4}\textsuperscript{2-}] = 0.35mM ) with BSA used as an additive. Scale bar 1\textmu m](image-url)
the formation of COD, the reason that the formation of urinary stone is inhibited and the reason that explains why though the urine is saturated with the CaOx\textsuperscript{37, 52, 79}, the urinary stone disease is not occurring to everyone.

We saw that at the supersaturation of 38.06, the competition of the templating effect of BSA suppressed the supersaturation driven interfacial mismatch effect. However, once the concentration is set to $[Ca^{2+}]=[C_2O_4^{2-}]=0.75mM$, the supersaturation is 145.93, which is much higher than 38.06. Under such conditions, the supersaturation driven interfacial mismatch effect totally controls the crystallization process. As presented in Fig. 5-5, the SEM pictures show that at the supersaturation of 145.93 with the BSA used as additive, both the COD and COM are disordered and distorted. It is clear that the high supersaturation\textsuperscript{10, 13, 47, 65, 69, 71, 73} results in the disordered crystal morphology. This means that perfect crystal morphology is hard to get at very high supersaturation.

![SEM micrograph of co-existence of COM and COD crystals](image)

Fig. 5-5. SEM micrograph of co-existence of COM and COD crystals, obtained from a solution at high concentration ($[Ca^{2+}]=[C_2O_4^{2-}]=0.75mM$) with BSA used as an additive. Scale bar 10μm
high supersaturations.

5.3 Conclusion

The SEM experiments have confirmed the findings deduced from the nucleation kinetics study. Here, the SEM pictures provide direct proof that BSA can inhibit the nucleation of CaOx crystal, and can increase the supersaturation range for obtaining better crystal assembly. In this experiment, all the factors that affect the crystal nucleation, growth and aggregation are discussed. Such as the supersaturation driven interfacial mismatch effect, the templating effect of the protein, and the effect of adsorption on the kink sites and embryo surface. The driving force for nucleation is the supersaturation, which can produce the supersaturation driven interfacial mismatch effect; the substrate can lower the energy barrier for nucleation, however, it can also reduce the effective surface for the growth units to be incorporated to the embryo surface. The additives such as protein can be adsorbed on the kink site, thus increasing the kink site energy barrier. These factors compete with each other. Consequently, the BSA influences not only the nucleation process, but also the crystal morphology.

The SEM pictures have also shown that the presence of BSA leads to the formation of COD. The medical benefit of the formation of COD rather than COM has been extensively discussed both in this part and in the previous chapter.
CHAPTER SIX

Discussion and Conclusion

6.1 Results and Discussion

In order to establish a better understanding of the crystallization of CaOx crystals under the effect of the protein BSA, based on a newly found nucleation theory, both the nucleation kinetics, and the structural synergy between the biomineral and the biosubstrate were examined in this study.

Armed with the newly founded nucleation theory, the nucleation kinetics of CaOx crystals has been studied. From the experimental results, we find that the BSA favors the formation of COD crystals, which are absent in the absence of the BSA. In addition, the way the BSA influences the nucleation kinetics has also been discussed. First, the presence of BSA lowers the nucleation energy barrier, thus promoting the crystal nucleation. Second, during the nucleation process, the BSA adheres to the kink sites and/or the embryo surfaces, thus increasing the kink energy barrier, and slowing down the crystallization. In all, from the experimental data, we understand that the BSA prolongs the CaOx nucleation process. This is achieved by the nucleation induction time increase. From the nucleation kinetics study, we also deduce that the
protein can enlarge the supersaturation range to achieve a better crystal morphology. This conclusion has also been confirmed by the crystal morphology study using SEM.

We also discussed the possible role of the albumin in treating the kidney stone. We know that COD is less likely to adhere to the urinary cells and tubes, and is less harmful to the kidney organ. In addition, the induction time increase makes the crystals to be more easily propelled out by urine. These three factors lead to the conclusion that the albumin plays a positive effect on preventing the urinary disease.

The most exciting result of this study is that with the newly founded nucleation theory, the nucleation kinetics and the crystal morphology have been interconnected and discussed together. The method of this study can also be used to investigate how the other proteins, lipids and organic matrix influence the urinary stone disease. Moreover, it can also be further developed to study all the nucleation kinetics of the biological crystals. In future, this work can be extended to investigate more complicated phenomena in nature, and more useful findings can be obtained.

However, though many achievements have been made in this study, some other questions have been raised, which await satisfactory answer. First, in this study, only the protein, albumin, is added and no other protein is considered. This raises the question about the possible role of other proteins and the possible combined effect of other proteins with albumin. Second, in real situation, there are many other ions in the kidney and urinary system. So, during the nucleation study, all of these ions should be considered. Third, in this study, we have not considered other organic matrix. The lack of other organic matter, for certainty, can make the study incomplete. Last, the nucleation, growth and assembly processes all happen in the urinary system, while, in
this study, structural information of kidney has not been considered. Though we have obtained much information about the substrate effect on CaOx crystallization, it is far from enough to understand the situation prevailing in the kidney, urinal tube and organs. Therefore, the nucleation theory must be advanced to make these points clearer. Moreover, though the sodium chloride is studied in the CaOx crystallization, its combinational effect with the albumin is still not clear. In other words, though some progress has been made in studying the urinary stone and protein, this study has also put forward some questions, which need satisfactory answers.

### 6.2 Recommendation for Further Research

The study on the effect of albumin on CaOx crystal nucleation, with the aid of the newly found nucleation theory, has been carried out for the first time. The results are very encouraging. However, this project is only a preliminary study. Further research is recommended to clarify the following aspects.

1. How do the other component in the urinary stone, such as the ions, proteins and lipids influence the CaOx crystal formation should be investigated. The combined role of other proteins, matrix and the kidney environment in the crystallization process makes the above question much complicated.

2. The protein has complex structure, so how does it affect the nucleation and by what means need to be answered. Whether the protein is denatured and to what extent it has been denatured are still unknown. In addition, if the protein is in the denatured state, what is its effect on CaOx crystallization?
3. Other inorganic components of the urinary stones, such as hydroxyapatite and carbonate apatite, should also be introduced into the crystal and studied.

4. Though in this study, the possible role of albumin in the formation of the urinary stone has been pointed out; it is still a long way to find a possible and reliable method to utilize this knowledge in medical treatment.
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