

Growth of monosodium urate monohydrate crystals: effect of cartilage and synovial fluid components on in vitro growth rates

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SUMMARY The effects of cartilage and synovial fluid components such as proteoglycans, chondroitin sulphate, hyaluronic acid, phospholipids, and albumin on the growth kinetics of monosodium urate monohydrate (MSUM) crystals were investigated. MSUM seed crystals were added to supersaturated sodium urate solutions, and the rate of decrease in the concentration of growth medium was used as a measure of the growth rate. A second order dependence of growth rate on supersaturation was found, and growth rate constants were determined with an integrated form of the growth equation. The additives, hyaluronic acid, proteoglycan monomer and aggregate, and phosphatidylserine, had no significant effect on the growth rate constant. Chondroitin sulphate and phosphatidylcholine increased the growth rate constant, possibly by promoting further nucleation in the growth medium. Albumin significantly inhibited MSUM crystallisation. The possible implications of these findings on in vivo MSUM crystallisation are discussed.

Key words: seeded growth technique, crystallisation accelerators, crystallisation inhibitors.

The joints in acute and chronic gouty arthritis are characterised by the presence of monosodium urate monohydrate (MSUM) crystals in the synovial fluid or on the surface and within the joint cartilage and synovium.^{1,2}

Hyperuricaemia alone is not sufficient for the crystallisation of MSUM, and other factors must therefore be involved in the formation of MSUM crystals. Little is known, however, about the physicochemical conditions necessary for the formation of MSUM crystals in articular cartilage and other sites in vivo. Factors such as local temperature or pH changes in joints cannot explain all the observed patterns of MSUM crystallisation.

Morphological and biochemical changes in cartilage and other connective tissues due to altered connective tissue metabolism, trauma, degenerative joint disease, and aging have been associated with the deposition of MSUM and calcium pyrophosphate dihydrate crystals in these tissues.³⁻⁵

The increased incidence of MSUM deposition and gouty arthritis with aging may be related to the

changes that occur in cartilage with advancing age, such as the significant alterations in the relative proportions of the glycosaminoglycans⁶ and the increases in the intracellular and extracellular lipid content of articular cartilage.⁷

It remains uncertain as to whether crystal deposition is a primary factor leading to joint damage in osteoarthritis or alternatively, whether osteoarthritis may lead to crystal deposition, as there is evidence to support both alternatives.^{4,8} The progressive degeneration of articular cartilage in osteoarthritis is characterised by a marked loss of glycosaminoglycans and proteoglycans.⁶

Although it is thought that cartilage is probably initially the principal area of deposition of MSUM crystals, crystals may also form directly in the synovial fluid.¹ Therefore, changes in the composition of synovial fluid could play a part in the crystallisation of MSUM in this fluid. Increased amounts of proteins (albumin and globulin) and lipids have been found in gouty and other pathological synovial fluids.⁹⁻¹²

It has been proposed that changes in the structure and composition of the connective tissues might predispose these tissues to crystal deposition by

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reducing inhibition to crystallisation or activating promoters of nucleation.¹³ Although there have been several studies of the factors affecting the solubility¹⁴⁻¹⁸ and nucleation¹⁹⁻²¹ of MSUM, the factors which may influence the crystal growth of MSUM are poorly understood.

The kinetics of crystallisation of MSUM have been studied,²²⁻²⁵ and several dyes, serum albumin, synovial fluid, and sodium heparin were shown to have an inhibitory effect on the growth rate of MSUM.

It has been suggested that alterations in the structure and composition of connective tissues, for example, the increased or decreased levels of the macromolecular components of cartilage in aging or osteoarthritis, may predispose these tissues to crystal deposition. Hence our studies have been directed towards determining the effects of cartilage and synovial fluid components such as proteoglycans, chondroitin sulphate, hyaluronic acid, phospholipids, and albumin on the growth kinetics of MSUM. The possible implications of our findings on *in vivo* MSUM crystallisation are discussed.

Materials and methods

PREPARATION AND CHARACTERISATION OF MSUM

Crystals of MSUM were grown using a slight modification of the method of Denko and Whitehouse.²⁶ A solution of uric acid (Sigma Chemical Co) and sodium hydroxide at 55°C and pH 8.9 was filtered through a 0.22 µm Millipore filter while hot. The filtrate was left to stand overnight at room temperature. The crystals were rinsed several times with cold (4°C) distilled water and dried at 60°C for 12 h in a circulating hot air oven. Samples of MSUM were ground in a glass mortar and pestle, and the batch of MSUM crystals to be used as seed crystals in growth experiments was passed through a number 50/80 US standard sieve (mesh size 180-300 µm) in an attempt to obtain a greater degree of size uniformity of seed crystals.

The crystals were characterised by the previously described methods of *x* ray diffraction, differential scanning calorimetry, scanning electron microscopy, and three point BET surface area analysis.²⁷

SATURATION SOLUBILITIES

Excess MSUM (200-500 mg) in 100 ml distilled water was equilibrated at 37°C in a shaking water bath. After filtration of an aliquot through a 0.22 µm Millex-GS (Millipore Corp) disposable filter unit the filtrate was assayed for the MSUM concentration by measuring the absorbance of the solution at 292 nm with an ultraviolet recording spectrophotometer

(Beckman, model 24). Sampling was terminated when three consecutive assays gave identical results (after about 12 h).

The solubility of MSUM at 37°C in the presence of the following additives was determined as described above: chondroitin sulphate 0.2-0.6 g/l (sodium salt, from whale or shark cartilage, 99%; Sigma Chemicals); hyaluronic acid 0.1-0.4 g/l (from human umbilical cord; Sigma Chemicals); albumin 0.1-1.0 g/l (fraction V from bovine serum; Sigma Chemicals); proteoglycan aggregate 0.2-1.0 g/l (A1 fraction); proteoglycan monomer 0.1-1.0 g/l (A1D1 fraction).

CRYSTAL GROWTH

A 5 g/l supersaturated solution of MSUM in water (pH 7.4) was prepared and 50 ml of the solution filtered through a 0.22 µm Millipore filter into a covered glass reaction vessel and equilibrated in a water bath at 37°C. An all glass stirrer with two paddles was lowered through a central port in the cover to a constant depth and rotated at 200 rpm by means of a stirrer motor (Fisher Stedi Speed stirrer). To the supersaturated solutions in the reaction vessel were added either 40 mg or 50 mg of seed crystals. Aliquots of 0.3 ml of growth medium were withdrawn at predetermined time intervals up to 360 min, filtered through a 0.22 µm Millex-GS disposable filter unit, diluted, and analysed immediately for MSUM content by measuring the absorbances at 292 nm.

EFFECT OF ADDITIVES

Quantities (given in parentheses) of the following additives were dispersed in 50 ml of a 5 g/l supersaturated MSUM solution: chondroitin sulphate (10, 20, 30 mg), proteoglycan monomer (5, 10 mg), proteoglycan aggregate (10, 20, 50 mg), albumin (100, 200 mg), hyaluronic acid (10, 20 mg).

Liposomal suspensions of phosphatidylcholine (10, 20, 30 mg) and phosphatidylserine (10, 20 mg) in 50 ml of a 5 g/l supersaturated MSUM solution were prepared by ultrasonication (ultrasonic cleaner; Mettler Electronics) a dispersion of the phospholipids in water.

The solutions in the reaction vessel were equilibrated at 37°C for 30 min, stirring constantly at a stirrer speed of 200 rpm. Seed crystal amounts of 40 mg were added, with the exception of experiments using the additive, hyaluronic acid, where 50 mg of seed crystals was added to the supersaturated solution. Aliquots of 0.3 ml of growth medium were withdrawn, filtered, and analysed for MSUM content as described previously.

On completion of sampling, the growth medium from each experiment was filtered through a 0.22

μm filter, the crystals on the filter dried at 60°C , and subjected to scanning electron microscopic examination and powder x ray diffraction.

SODIUM AND POTASSIUM CONTENTS OF ADDITIVES

Solutions of 0.1 mg/ml of chondroitin sulphate, hyaluronic acid, proteoglycan monomer, or proteoglycan aggregate in distilled water were prepared, and the absorbances of the solutions measured on an atomic absorption spectrometer (Jerald Ash) at wavelengths of 585 nm for sodium and 765 nm for potassium. The concentrations of sodium or potassium, or both, in the samples were determined from standard curves.

Results

x Ray powder diffraction patterns of all MSUM samples gave d values characteristic of MSUM.²⁷ Differential scanning calorimetry scans of MSUM gave an endothermic peak corresponding to the loss of one mole of water of hydration. Scanning electron micrographs of MSUM seed crystals showed the typical long, well formed needle shaped crystal habit of MSUM.²⁷

It is well documented that bacterial consumption of urates in solution is a problem, and previous studies of solubility, nucleation, or crystal growth rates of MSUM were carried out either under non-sterile or sterile conditions.^{20 21 23} Owing to the difficulties in maintaining sterility during crystal growth rate studies, we carried out stability studies to determine the length of time for which a non-sterile MSUM solution could be stored at 37°C without significant degradation. The results indicated that an MSUM solution prepared in filtered (0.22 μm filter) distilled water could be stored for up to 20 hours without significant loss of urate (less than 1.5% degradation). Hence all growth rate and solubility studies were completed within 6–12 h.

The saturation solubility of MSUM at 37°C , determined by interpolation of a van't Hoff plot,

was 1.379 g/l, which is within the range of values reported by other workers.^{20 22 24}

Table 1 shows the saturation solubilities of MSUM at 37°C in the presence of differing quantities of chondroitin sulphate, hyaluronic acid, proteoglycans, and albumin. These values were subsequently used in the determination of rate constants for MSUM crystal growth in the presence of additives.

Increasing quantities of chondroitin sulphate resulted in small decreases in MSUM solubility from 1.379 g/l (no additive) to 1.329 g/l (0.6 g/l chondroitin sulphate). Laurent also showed a similar effect,²⁸ but Katz and Schubert attributed the decrease in solubility shown by Laurent to the uricase action of bacterial contaminants in the solutions.¹⁶ Katz and Schubert reported a small increase in MSUM solubility in an 80 mg/ml solution of chondroitin sulphate in phosphate buffer containing 0.13 M sodium chloride.¹⁶

Chondroitin sulphate obtained commercially is a mixture of chondroitin-6-sulphate and chondroitin-4-sulphate and was found by atomic absorption spectrometry to contain 5.6% w/w sodium. The decrease in MSUM solubility observed may be due to the suppression of solubility by the sodium in the chondroitin sulphate samples. We found the saturation solubility of MSUM in the presence of 154 mM sodium chloride at 37°C to be 0.08 g/l, which is within the range of reported values.^{15 22 24}

Hyaluronic acid, albumin, proteoglycan monomer and aggregate all caused very small increases in MSUM solubility (Table 1). The commercial hyaluronic acid from umbilical cord contained 0.11% w/w sodium and 6.3% w/w potassium. The proteoglycan monomer and aggregate contained 5.8% w/w and 5.2% w/w sodium, respectively. Perricone and Brandt showed that hyaluronic acid and low concentrations of proteoglycan aggregate had a negligible effect on MSUM solubility.¹⁷ Higher concentrations of proteoglycan monomer also had no effect on urate solubility, but high concentrations of proteoglycan aggregate either as the sodium or potassium

Table 1 Saturation solubility of MSUM in the presence of additives at 37°C

Additive	Saturation solubility of MSUM* (g/l) in the presence of additive concentrations (g/l):				
	0.1	0.2	0.4	0.6	1.0
Chondroitin sulphate	—	1.358	1.347	1.329	—
Hyaluronic acid	1.392	1.387	1.401	—	—
Albumin	1.382	1.391	1.397	—	1.395
Proteoglycan monomer	1.402	1.397	1.399	—	1.401
Proteoglycan aggregate	—	1.408	1.397	—	1.403

*Mean of three determinations.

salt enhanced urate solubility. The potassium in the preparation, however, rather than the proteoglycans, may account for the effect of proteoglycan aggregate on urate solubility.¹⁸

Solutions with a supersaturation MSUM concentration of 5 g/l showed immediate growth when seeded with 40 mg MSUM crystals. A typical growth rate curve is shown in Fig. 1 (control).

The rate of crystallisation of MSUM in supersaturated solutions would be expected to follow an equation of the form:

$$R_g = K' (C - C_s)^2 \quad (1)$$

where R_g is the rate of growth (decrease in concentration per unit time), K' is the growth rate constant and $K' = K_0 S$ where K_0 is the overall growth rate constant and S is the surface area of seed crystals, C is the solution concentration at various time intervals, and C_s is the saturation solubility of MSUM.

The excellent linearity (correlation coefficients of 0.97 or greater) of plots of the integrated form of equation (1) (equation 2) confirms the applicability of equation (1) at a supersaturation concentration of 5 g/l.

$$\frac{1}{C - C_s} - \frac{1}{C_i - C_s} = K't \quad (2)$$

where C_i is the initial supersaturation concentration and C and C_s are as defined above.

Values of K' were determined from the slopes of the lines and K_0 from K'/S . The mean surface area of the seed crystals was 1.452 m²/g ($n=3$, range: 1.417–1.548 m²/g). The mean growth rate constants, K' and K_0 , for growth of MSUM from a solution of initial supersaturation concentration 5 g/l and seeded with 40 mg MSUM crystals were 45.7×10^{-5} l/g·min (range: 42.6×10^{-5} – 48.4×10^{-5} l/g·min) and 7.8×10^{-3} l/g·min·m² (range 7.3×10^{-3} – 8.3×10^{-3} l/g·min·m²), respectively.

It has been shown previously that the addition of sodium ions suppresses the saturation solubility of MSUM. Since the additives, chondroitin sulphate, hyaluronic acid, proteoglycan monomer, and proteoglycan aggregate, contain sodium or potassium, or both (see above), the effect on the growth rate constants of the concentrations of sodium or potassium present in the maximum quantities of additives used in growth experiments was determined. The growth rate constants in the presence of either 1.6 mg Na⁺ or 1.3 mg K⁺ added to 50 ml of a supersaturated MSUM solution were determined and were not significantly different from the growth rate constants in the absence of these concentrations of Na⁺ or K⁺. Therefore, it is apparent that the small additional contribution made by the Na⁺ or K⁺ present in the additives to the overall high

concentration of Na⁺ in the supersaturated MSUM solutions was insufficient to cause any significant change in the growth rate constant.

The effect of additives on the growth rate constants is given in Table 2. The growth rate constants for the growth of MSUM in the presence of additives, $K'(\text{add})$, were determined from the slopes of plots of equation (2), where C_s was taken to be the saturation solubility of MSUM in the presence of the same concentration of additive (Table 1). The overall growth rate constants in the presence of additives, $K_0(\text{add})$, were calculated from $K'(\text{add})/S$. The ratio of the overall growth rate constant in the presence of additive, $K_0(\text{add})$, to the overall growth rate constant in the absence of additive (control), K_0 , was calculated, where K_0 values under identical conditions of supersaturation concentration and sodium or potassium ion concentration were employed. If the ratio $K_0(\text{add})/K_0$ was greater than or less than unity the additive under study either increased or decreased the growth rate constant for growth of MSUM, respectively. Statistical analyses were performed using a one way analysis of variance followed by a Neuman-Keul's test. The level of significance was $p < 0.05$.

The results in Table 2 show that at the concentrations employed in these studies the additives hyaluronic acid, proteoglycan monomer, proteoglycan

Table 2 Effect of additives on the growth rate constant of MSUM at 37°C

Additive	Amount of additive in 50 ml supersaturated solution (mg)	$K_0(\text{add})/K_0^*$
Hyaluronic acid	10	0.97†
	20	0.94†
Chondroitin sulphate	10	1.55‡ (p<0.05)
	20	1.57† (p<0.05)
	30	1.66† (p<0.05)
Proteoglycan monomer	5	1.02‡
	10	1.05†
Proteoglycan aggregate	10	0.97†
	20	1.12†
	50	1.16†
Phosphatidylcholine	10	1.12† (p<0.05)
	20	1.33† (p<0.05)
	30	1.30† (p<0.05)
Phosphatidylserine	10	1.12†
	20	1.03‡

*Ratio of the overall growth rate constant in the presence of additive to the overall growth rate constant in the absence of additive (control).

†Mean of two determinations.

‡Mean of three determinations.

p<0.05, rate constant in the presence of additive was significantly different from the rate constant in the control.

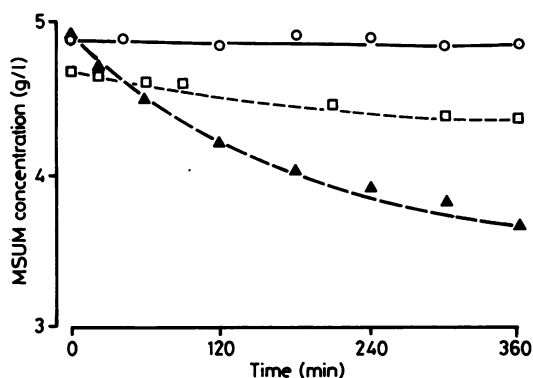


Fig. 1 Seeded growth of MSUM in the presence of albumin at 37°C and an initial supersaturation concentration of approximately 4.9 g/l. Solutions seeded with 40 mg seed crystals. Quantities of albumin added: ○, 200 mg; □, 100 mg; ▲, no albumin (control).

aggregate, and phosphatidylserine had no significant effect on the growth rate constant of MSUM. At concentrations of between 0.2 and 0.6 mg/ml chondroitin sulphate and phosphatidylcholine there was a significant increase in the MSUM growth rate constant.

The effect of albumin on the growth of MSUM is shown in Fig. 1. The rate constants for MSUM growth in the presence of albumin are not given because very poor correlations were obtained when plots of equation (2) were constructed. There was a significant reduction in the growth rate in the presence of 100 mg albumin, and 200 mg albumin caused almost complete inhibition of MSUM crystal growth.

After completion of growth experiments, both in the presence and absence of additives, the MSUM crystals were characterised by scanning electron microscopy and x ray diffraction. Scanning electron micrographs of MSUM crystals after growth in the presence of additives showed that the needle shaped crystal habit of MSUM was retained on completion of the growth experiments. The d values for all MSUM samples after growth either in the presence or absence of additives were identical to d values obtained for the original MSUM seed crystals, confirming that there was no alteration in the crystalline structure of MSUM after growth.

Discussion

In this study we have used the seeded growth technique developed by Lam Erwin and Nancollas.²² Thus the process of nucleation is

avoided and only the subsequent crystal growth kinetics are observed. The seeded growth technique has been found to be highly reproducible and has been used in the study of the crystallisation kinetics of a great many compounds.

The results of our crystal growth experiments in the absence of additives correspond quite closely to those obtained by Lam Erwin and Nancollas,²² who found that the MSUM growth rate followed the square of the relative supersaturation. Fiddis *et al*, however, showed a much stronger dependence of growth rate of MSUM on supersaturation.²⁵

The increase in the MSUM growth rate constant in the presence of chondroitin sulphate was not due to a decrease in the solubility since the highest concentration of chondroitin sulphate used in the growth experiments (0.6 mg/ml) decreased the solubility of MSUM by less than 4% (Table 1). Since chondroitin sulphate is not acting as a growth accelerator by suppressing the solubility it is possible that this large, negatively charged molecule may act by promoting further nucleation. The mechanism for this, however, is unclear. If additional nuclei were formed in solution, this would provide a greater surface area of seed material available for growth, and would enhance the growth rate. This hypothesis of enhancement of nucleation by chondroitin sulphate would contradict the findings of Tak *et al*, who observed no effect of chondroitin-4-sulphate or chondroitin-6-sulphate at concentrations of 1 mg/ml on the nucleation of 5 mM urate solutions.²⁰ We have employed solutions of much higher urate concentration, however, than in this latter study. Katz studied the crystallisation of urate from supersaturated solutions and showed that chondroitin sulphate had no significant inhibitory effect on the crystallisation of MSUM,²⁹ but whether any enhancement of crystallisation was observed was not discussed. It is interesting to note that chondroitin sulphate is a normal constituent of synovial fluid, and raised concentrations of this component have been found in some pathological fluids.¹⁰ The implication of our findings on the *in vivo* crystallisation of MSUM could be that if the chondroitin sulphate levels in synovial fluid became raised, the increased amount of chondroitin sulphate might be an important factor in the promotion or acceleration of the crystallisation of MSUM in the synovial fluid of a hyperuricaemic individual.

In our growth experiments a mixture of unknown composition of the two isomers of chondroitin sulphate, chondroitin-4-sulphate and chondroitin-6-sulphate, was used, and it is possible that the two isomers could have quite different effects on MSUM growth. There is some evidence that biochemical

changes in cartilage due to factors such as aging or joint disease could predispose cartilage to crystal deposition. In both aging and osteoarthritis the proportion of chondroitin sulphate is decreased. Hence it seems unlikely that chondroitin sulphate is a major contributing factor in the deposition of MSUM in cartilage.

The two phospholipids studied, phosphatidylcholine and phosphatidylserine, also increased the growth rate constant of MSUM. At the concentration levels of phosphatidylserine used in the growth experiments the increase in the growth rate constant was not significant. The phospholipids added to the supersaturated growth medium were added as suspensions of phospholipid vesicles or liposomes. It is possible that the increase in the MSUM growth rate constant in the presence of phosphatidylcholine was due to the liposomes providing additional nucleation centres, thus increasing the surface area available for growth.

There is a significant increase in the intracellular and extracellular lipid content of articular cartilage with age, and extracellular lipids are prominent in the surface layers of cartilage. These lipids consist of triglycerides, cholesterol or cholesterol esters, phospholipids, and glycolipids. Normal synovial fluid contains small amounts of phospholipids and cholesterol, and synovial fluid from patients with rheumatoid arthritis and osteoarthritis shows increased amounts of phospholipids, cholesterol, and neutral lipids. The phospholipid composition of normal synovial fluid is similar to that of plasma, with phosphatidylcholine being the major constituent. It is possible that the raised levels of phospholipids in aged or diseased cartilage and synovial fluid could accelerate the growth of MSUM crystals, resulting in MSUM deposition in these tissues.

Albumin caused a significant decrease in the MSUM growth rate at a concentration of 2 mg/ml, probably due to adsorption of the albumin molecules onto the MSUM crystal surface with subsequent poisoning of the active growth sites on the crystal surface. Albumin is known to adsorb readily onto glass surfaces,³⁰ and therefore there was probably a significant loss of albumin from the growth medium due to non-specific adsorption of albumin molecules onto the glass reaction vessel and stirrer. This would result in reduced concentrations of inhibitor in solution. Fiddis *et al* found that albumin at a concentration thought to approximate that in synovial fluid (10 mg/ml) increased the crystallisation time by a factor of about four,²⁵ which is not a large effect. In a similar type of experiment Katz found that 100 mg/ml albumin

failed to inhibit urate crystallisation significantly.²⁹ We have shown that 4 mg/ml albumin caused almost complete inhibition of crystallisation, a much greater effect than that shown by Fiddis *et al*.²⁵ Part of the reason for these differences in effect may be due to the problem of adsorption of albumin onto the glass or plastic surfaces of growth apparatus. Albumin levels have been found to be raised in the synovial fluids of gouty patients. Thus although we have shown that albumin is an inhibitor of urate crystallisation, raised levels of this inhibitor cannot explain the precipitation of MSUM crystals in gouty synovial fluids.

Tak *et al* found that hyaluronic acid at a concentration of 0.065 mg/ml, approximately one tenth the normal synovial fluid level, caused heterogeneous nucleation on the walls of the vials.²⁰ Our work shows that at a concentration of 0.4 mg/ml hyaluronic acid had no significant effect on the overall growth kinetics of MSUM.

At concentrations of proteoglycan monomer between 0.1 and 0.2 mg/ml, and of proteoglycan aggregate between 0.2 and 1.0 mg/ml we found no significant effect of either monomer or aggregate on the growth kinetics of MSUM. Blumenthal *et al* studied the effect of bovine nasal cartilage proteoglycan aggregate and proteoglycan monomer on the direct precipitation of hydroxyapatite from low concentration calcium phosphate solutions.³¹ They found that, although the time of onset of apatite formation was increased by proteoglycans, the subsequent growth kinetics were not affected. This latter observation is similar to our findings that proteoglycans did not significantly alter the growth kinetics of MSUM. At the higher concentrations of proteoglycans found in cartilage (15–60 mg/ml)³² the growth kinetics of both MSUM and apatite could be affected.

Many macromolecules have the ability to adsorb at interfaces, and in future work we intend to determine the extent of adsorption of the additives to MSUM crystal surfaces.

It is evident that the effects of the different cartilage and synovial fluid components on the seeded growth of MSUM from supersaturated solutions are very complex. Our results would suggest that alterations in the concentration of some components as a result of aging, pre-existing disease, or altered matrix metabolism could have significant roles in the growth of MSUM crystals in joints. Crystal deposition, however, may be a process involving the interplay of several factors. For instance, alterations in more than one cartilage or synovial fluid component may be necessary before MSUM growth proceeds. Factors such as local

temperature and pH, concentrations of ions such as magnesium, calcium, sodium, pyrophosphate, etc. water content of cartilage, and other cartilage components not studied to date, such as glycoproteins and keratan sulphate, may also influence the deposition and growth of MSUM crystals.

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