Scanning Electron Microscopy

Volume 1986
Number 1 *Part I*

Article 15

4-8-1986

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SCANNING ELECTRON MICROSCOPY/1986/I (Pages 151-156)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 USA

0586-5581/86$1.00+05

THEORY FOR CALCIUM-PHOSPHATE CRYSTAL FORMATION IN TISSUE FROM SCANNING ELECTRON MICROSCOPE DATA

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(Received for publication June 19, 1985, and in revised form April 08, 1986)

Abstract

Scanning electron microscope (SEM) morphological analysis combined with energy dispersive characteristic x-ray analysis provides insight into the mechanism of biological mineralization. A time series of tissue micrographs and mineralization measurements can permit the determination of the mineralization kinetic behavior and is the basis upon which a computer model has been devised. The computer model is constructed from fundamental principles of crystal nucleation and precipitation theory. Various general forms of the model are tested against the laboratory data for goodness-of-fit using the least squares method, and two models are found to be acceptable. Both of the acceptable models involve inhibition of the mineralization process which has a reaction order ranging from one to two. A third model involving constant nucleation rate must be rejected.

Having established working first principle models for the mineralization process, one can compute a constant number of nucleation sites and a supersaturation value for calcium in various mineralized tissues such as the spongiosa and fibrosa of heart valve leaflet implants. These quantities are determined and used in discussing a general theory for biomineralization which emphasizes therapeutic considerations.

Key words: Biological calcification, mineralization, biological crystals, calcification, calcium phosphate crystals, crystal formation, crystal theory, mineralization model.

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Introduction

The purpose of this study is to formulate an appropriate mathematical model that explains the biological mineralization data presented in our earlier paper (Nelson et al., 1985). In that work, glutaraldehyde pretreated porcine heart valve leaflets implanted in a rat model system and subsequently removed at various times ranging from 1 day to 56 days post-implantation (Levy et al., 1983) were examined under scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy. The SEM morphological and semi-quantitative x-ray analyses were used to measure the relative amounts of deposited calcium in the spongiosa and fibrosa regions of the valve tissue system. The data from this study are utilized throughout the theoretical formulation and analysis presented here. The numbers for calcium counts are the normalized characteristic x-ray signal intensities and do not equate directly to absolute concentrations of the ion.

We begin with a review of the data and a discussion of the calcification process based on some relevant work on biological mineralization and then continue to deduce the appropriate data fitting functions. After comparison of different curve fitting results, some mechanistic interpretations of the mineralization process will be discussed.

List of Symbols

n	=	number of calcium ions per unit volume
n_s	=	saturation number of calcium ions per unit volume
n_{ss}	=	supersaturation number of calcium ions per unit volume.
$n(t)$	=	time varying number of calcium ions per unit volume
n_o	=	number of calcium ions per unit volume at t= 0 when crystalization is initiated.
$n_p(t)$	=	number of calcium ions per unit volume which have precipitated at time t
J	=	rate of nucleation of calcium
J_{max}	=	maximum rate of nucleation of calcium
$N(t)$	=	time varying number of nucleation sites per unit volume
N_I	=	constant concentration of nucleation sites due to inhibition
ΔG_o	=	adsorption energy
k_B	=	Boltzmann's constant
T	=	temperature in Kelvin
x	=	reaction order
k	=	crystal growth rate constant

Development of the Model

The data used in this analysis were obtained from scanning electron microscopy characteristic x-ray detection for calcium in representative regions of the spongiosa and fibrosa layers in porcine heart valve tissue. Several post-implantation time periods were sampled to obtain a time varying profile of tissue mineralization. For example, Figure 1 shows a cut surface of valve tissue implanted for 3 days. In contrast, Figure 2 shows another surface of a tissue implanted for 7 days where the increased degree of mineralization is apparent as small crystalline sites not found on the 3-day implant. By 21 days post-implantation, the spongiosa has become heavily mineralized (Figure 3) and an x-ray dot map for calcium in the same tissue confirms this observation (Figure 4). Dot maps for phosphorus have an appearance similar to calcium. The x-ray data from the various tissue specimens are normalized against a copper standard and tabulated as the normalized counts of calcium as a function of post-implantation time. Table 1 shows the Ca counts for time periods of 1, 3, 7, 14, 21, 28, and 56 days. Two data columns are shown, one for spongiosa and one for fibrosa, and these data are examined from the perspective of crystallization theory.

The biological mineralization process involves crystallization in an ionic solution in the environment of cells and tissue. The generation and growth of calcium-phosphate crystals may be described in terms of four stages which will be addressed using calcium as the example.

Supersaturation

The supersaturation of the calcium ion in tissue fluid is a necessary condition for its crystallization. For the heart valve tissue implants from which the data for this analysis are obtained, the condition of calcium supersaturation is likely to be reached within a day following implantation. Let n be the number of calcium ions per unit volume, let n_s be the saturation concentration of calcium ions, and let n_{ss} be the supersaturation concentration which is always larger than n_s. We will use the word concentration to mean number per unit volume. For any point in time we can define an instantaneous calcium concentration as n(t), and at t=0 when crystallization is initiated the calcium concentration will be denoted by $n_o = n(o)$. Hence we can write $n_{ss}(t) = n(t) - n_s$ where the initial supersaturation concentration is simply $n_o - n_s$.

Nucleation

Nucleation in crystal growth is the continuous formation and dissolution of ionic clusters in equilibrium with other local clusters (Zhdanov, 1965). The rate of nucleation J is derived generally from the law of mass action (Walton, 1967):

$$J = \frac{d}{dt} N(t) = J_{max} \exp(-\Delta G_o/k_B T) \quad (1)$$

where N(t) is the instantaneous concentration of nucleation sites per unit volume, ΔG_o is the adsorption energy and is defined as the difference in free energy for calcium in solution and in cluster, J_{max} is a constant dependent on the specific experimental conditions and represents the maximum nucleation rate at zero free energy difference, T is temperature in Kelvin, and k_B is Boltsmann's constant.

In biological mineralization, nucleation probably consists of adsorption of calcium on or within a protein substrate as in bone growth, for example. Factors which would tend to increase the rate of nucleation are low adsorption energy ΔG_o, strongly adsorbing sites, and good lattice matching between the deposit and the substrate. However, the presence of inhibitors may appreciably decrease the rate of nucleation. Although some body fluids are saturated with respect to minerals such as calcium-phosphates and other calcium salts, the uncontrolled separation of solid phases is usually inhibited. There is considerable evidence from both *in-vitro* and *in-vivo* studies that inhibitors of crystallization of calcium salts are involved in the process of normal and pathological calcification (Nancollas, 1977). Identifiable inhibitors are, for example, chondroitin sulfate, heparin, and certain metal ions of cadmium, magnesium, and zinc (Harris et al, 1969).

Inhibitors may be effective in limiting the concentration of nucleation sites; therefore, two cases must be considered:

(i) For the uncontrolled nucleation case,
$N(t) = Jt$

(ii) For the strongly inhibited case,
$N(t) = N_I$

where N_I is a constant concentration of nucleation sites.

Diffusion

In the process of crystallization, ions tend to move toward crystal sites by simple diffusion. Since the biomineralization characteristic time is long compared to diffusion times (days compared to seconds) the diffusion process is assumed to contribute negligibly to the time course of crystallization. Thus, the interaction between calcium and the crystal site is assumed to occur instantaneously.

Precipitation

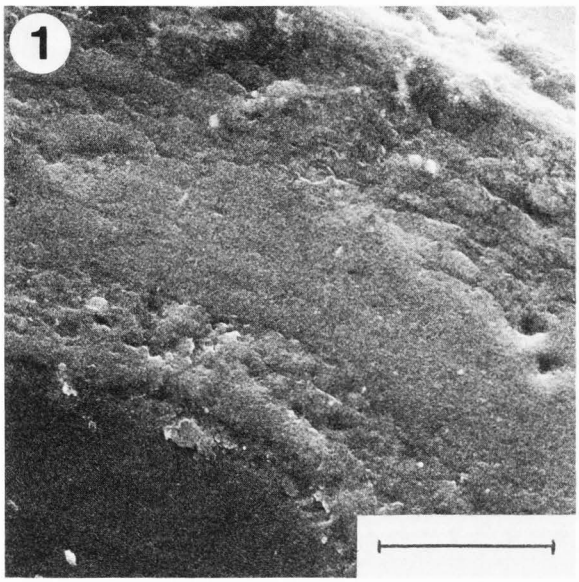
The process of precipitation (or crystal growth from seed crystals) is well established in comparison with the process of nucleation (Walton, 1967). The general form of the precipitation equation is,

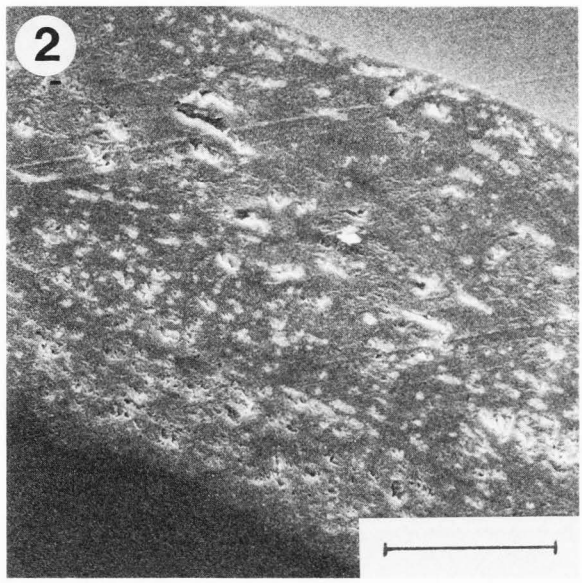
$$\frac{d}{dt} n(t) = -N(t)k[n(t)-n_s]^x \quad (2)$$

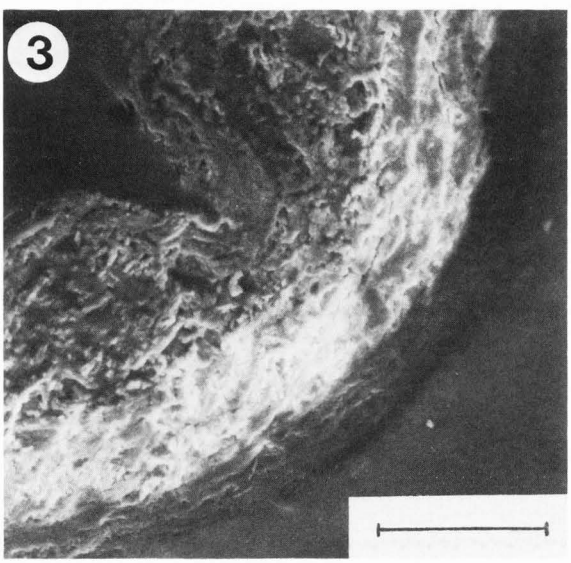
where n(t) is the instantaneous concentration of calcium in solution, N(t) is the instantaneous concentration of nucleation sites defined in equation (1), k is the crystal growth rate constant which depends on local environmental conditions and geometric factors such as crystal surface area, n_s is the saturation concentration of calcium and x is the reaction order which depends on the nature of the reactants and the environment. Typically, a diffusion controlled reaction would have x=1 whereas a reaction controlled by the crystal surface properties would have x=2.

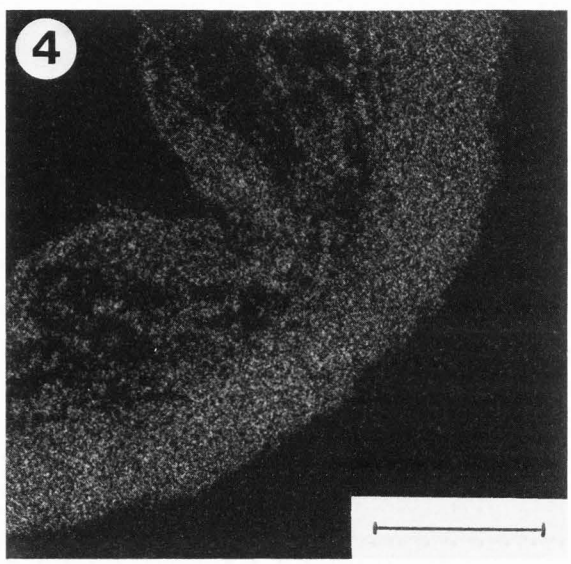
For the present study some of these factors can be specified or simplified. In particular, if the final size of a mineral crystal does not differ appreciably from the initial size, then the rate constant k is independent of geometric factors. Also, since there is no definitive data to establish the value of the reaction order x for calcium-phosphate mineralization, it is reasonable to assume simple situations such as $x = 1$ and $x = 2$, and it is frequently shown in other crystallizing systems that $x = 2$ (Walton, 1967).

These arguments are the basis for postulating three formulations of the biomineralization process: namely the heavily inhibited case with $x = 1$, the uncontrolled growth case with $x = 1$, and the heavily inhibited case with $x = 2$.









CASE 1: Crystal growth is heavily inhibited; reaction order is one.

$$N(t) = N_I \text{ and } x = 1.$$

From equation (2) we can write,

$$\frac{d}{dt} n(t) = -N_I k[n(t)-n_s].$$

Solving this equation and applying boundary conditions we obtain,

$$n(t) = n_s + (n_o-n_s) \exp(-N_I kt).$$

Therefore, the concentration of precipitated calcium is,

$$n_p(t) = n_o-n(t) = (n_o-n_s) [1-\exp(-N_I kt)]. \quad (3)$$

Fig. 1. Micrograph of porcine valve prepared 3 days post-implantation. There is little evidence of crystal formation at this early time. Bar = 50 μm.

Fig. 2. Micrograph of porcine valve prepared 7 days post-implantation. The bright patches are regions of calcium-phosphate crystallization. Bar = 50 μm.

Fig. 3. Micrograph of porcine valve prepared 21 days post-implantation. The bright band nearly 40 μm in width is the highly mineralized spongiosa tissue. Bar = 50 μm.

Fig. 4. Energy dispersive x-ray dot map for calcium in the same porcine valve interface shown in Figure 3. Again, the spongiosa tissue shows heavy mineralization. Bar = 50 μm.

CASE 2: Crystal growth is uninhibited with a constant nucleation rate; reaction order is one.

$$N(t) = Jt \text{ and } x = 1.$$

From equation (2) we can write,

$$\frac{d}{dt} n(t) = -Jtk[n(t)-n_s].$$

Solving this equation and applying boundary conditions, we obtain,

$$n(t) = n_s + (n_o-n_s)\exp(-Jkt^2/2).$$

Therefore, the concentration of precipitated calcium is,

$$n_p(t) = n_o-n(t) = (n_o-n_s)[1-\exp(-Jkt^2/2)] \quad (4)$$

CASE 3: Crystal growth is heavily inhibited; reaction order is two.

$$N(t) = N_I \text{ and } x = 2.$$

From equation (2) we can write,

$$\frac{d}{dt} n(t) = -N_Ik[n(t)-n_s]^2.$$

Solving this equation and applying boundary conditions we obtain,

$$n(t) = n_s + \frac{(n_o-n_s)}{1+N_Ikt(n_o-n_s)}.$$

Therefore, the concentration of precipitated calcium is,

$$n_p(t) = n_o-n(t) = (n_o-n_s) - \frac{(n_o-n_s)}{1+N_Ikt(n_o-n_s)}. \quad (5)$$

The three cases described above are formulated in equations (3), (4), and (5), and these are the equations which are to be compared with the experimental data to determine goodness-of-fit using the least squares method.

Curve Fitting Results

The three formulations are taken as model functions to perform least squares fitting to search for those coefficients which minimize the root-mean-square deviations from the experimental data. Linearized fitting functions are employed with the utilization of both grid search and gradient search methods (Bevington, 1969). The fitting functions are,

For Case 1: let $F_1 = A_1 [1 - \exp(-t/A_2)]$ (6)
with $A_1 = n_o-n_s$ and $A_2 = 1/N_Ik$ from equation (3).

For Case 2: let $F_2 = A_1 [1 - \exp(-t^2/A_2)]$ (7)
with $A_1 = n_o-n_s$ and $A_2 = 2/Jk$ from equation (4).

For Case 3: let $F_3 = A_1 - \frac{A_1}{1+tA_1/A_2}$ (8)
with $A_1 = n_o-n_s$ and $A_2 = 1/N_Ik$ from equation (5)

The computer printout results of plotting and fitting are shown in Figure 5 which compares fitting functions F_1, F_2 and F_3 to the actual data from Table 1. From these results it is seen that F_1 provides the best fit while F_3 fits nearly as well. F_2 fits poorly and is an unacceptable model especially in light of the likelihood that the crystallization process is an inhibited one. Thus we can reject the Case 2 model, equation (4). The models represented by Case 1 and Case 3 with equations (3) and (5) are acceptable and indicate that the crystallization process is indeed an inhibited reaction with reaction order of one or two or a combination of both.

Using the Case 1 fitting and the Case 3 fitting to determine optimal values for A_1 and A_2 it is found that,

Case 1	**Case 3**
For spongiosa tissue,	For spongiosa tissue,
$A_1 = n_o - n_s = 1.6$	$A_1 = 2.1$
$A_2 = 1/N_Ik = 8$ days	$A_2 = 20$ days
For fibrosa tissue,	For fibrosa tissue,
$A_1 = 1.0$	$A_1 = 1.3$
$A_2 = 4.9$ days	$A_2 = 8.3$ days.

It should be noted that the experimental data are relative, but if a calibration factor could be determined, the absolute concentration of calcification can be calculated. The crystal growth rate constant k is embedded in A_2 and is dependent on specific experimental conditions; therefore, A_2 can be used only for comparing experimental results under similar conditions. If k could be determined through some independent experiment, then N_I could be calculated, and this is desirable since a knowledge of the concentration of nucleation sites would elucidate the biophysical mechanism of mineralization. Even with these uncertainties, the constants A_1 and A_2 have an interpretation: A_1 represents the final intensity of mineralization; A_2 represents the characteristic mineralization time in units of days.

Conclusions

The experimental data for mineralization of bioprosthetic valve implants clearly show consistency with the general behavior of crystallization when the process is analyzed as a simple physicochemical one. The modelling demonstrates that the process proceeds with a constant concentration of nucleation sites in the tissue during the course of mineralization, and the process is likely to be controlled by inhibitors. The total reaction order x is likely to be between one and two. Because the biomineralization process is probably not diffusion limited, we would favor Case 3, the model with reaction order $x = 2$ corresponding to the surface limited situation. This is consistent with Walton (1967).

It is important to note that in pathological mineralization, therapeutic measures may be undertaken which would tend to decrease the total number of nucleation sites in tissue and/or slow the process of crystal growth. These changes would be reflected in the model parameters N_I and k in the A_2 coefficient, while the A_1 coefficient will probably be unaffected by therapeutic measures. Therefore, the Case 1 and Case 3 models may be useful in interpreting the results of future experiments concerning the treatment for pathological mineralization in human patients.

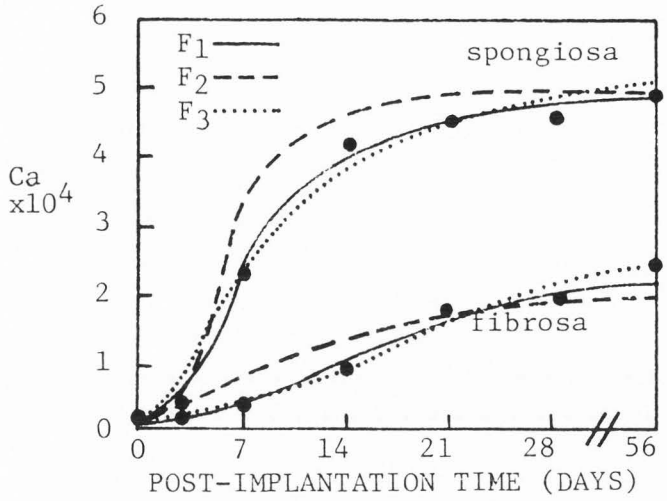


Fig. 5. Plot of the porcine valve Ca counts as a function of post-implantation time for spongiosa and fibrosa tissues separately. The three fitting functions F_1, F_2 and F_3 have been computer fit to the data points showing that F_1 and F_3 fit reasonably well. Data points were taken directly from Table 1.

Table 1
Experimental Data

Time (Days)	Ca×10⁴ Spongiosa	Ca×10⁴ Fibrosa
1	0.1	0.01
3	0.3	0.1
7	2.4	0.3
14	4.1	1.0
21	4.5	1.8
28	4.6	1.8
56	4.9	2.4

The data were taken from the paper "Scanning Electron Microscope Study of the Pathophysiology of Calcification in Bioprosthetic Heart Valves" (Nelson et al., 1985). The values for Ca counts are computed directly from the characteristic x-ray count data which have been normalized against a copper standard. The numbers are related to actual concentration but cannot be equated to it due to geometric and efficiency factors in the data collection method.

Acknowledgements

The author wishes to extend special thanks to the W.M. Keck Foundation for its general support, to Dr. Yongsheng Chao for his technical assistance and computer analysis, and to Elizabeth Connors for her expert preparation of the manuscript.

References

Bevington PR. (1969). *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill Book Co., New York, pp. 99–108 and 208–210.

Harris AF, Cotty VF, Barnett L, Seiman A. (1969). The Effect of Sulfated Mucopolysaccharides on Calcification. Arch. Intl. Pharmacodyn., **181**, #2, 489–498.

Levy RJ, Schoen FJ, Levy JT, Nelson AC, Howard SL, Oshry LJ. (1983). Biologic Determinants of Dystrophic Calcification and Osteocalcin Deposition in Glutaraldehyde-Preserved Porcine Aortic Valve Leaflets Implanted Subcutaneously in Rats. Am. J. Pathol., **113**, 143–155.

Nancollas GH. (1977). The Mechanism of Biological Mineralization. J. Crystal Growth, **42**, 185–193.

Nelson AC, Schoen FJ, Levy RJ. (1985). Scanning Electron Microscope Study of the Pathophysiology of Calcification in Bioprosthetic Heart Valves. Scanning Electron Microsc. 1985; I: 209–213.

Walton AG. (1967). Nucleation (Chapter 1) and Precipitation and Growth Kinetics (Chapter 2), In: *The Formation and Properties of Precipitates,* Interscience Publications, John Wiley & Sons, New York, pp. 1–74.

Zhdanov GS. (1965). Formation and Growth of Crystals, In: *Crystal Physics*, Academic Press, New York, pp. 365–379.

Discussion with Reviewers

Reviewer I: There is evidence that porcine heart valve leaflets which have not been pretreated with glutaraldehyde do not function well *in vivo*, but they do not calcify either. What does this suggest about the site of calcification?

Author: Apparently, the reaction of glutaraldehyde with the porcine tissue has the effect of creating sites for calcification wherever dialdehyde linkages are potentially formed. It may be possible to post-treat the glutaraldehyde fixed tissue with agents like SDS that may block or remove the reactive groups.

Reviewer I: How does this theoretical model for calcification in bioprosthetic heart valves compare to other forms of physiologic and pathologic calcification? Information on the change with time of calcific crystal domain size could help distinguish between increased calcification caused by crystal growth or that caused by crystal nucleation. Would the author comment on these points?

Author: Since our Ca/P ratios are similar to those for biological bone formation, we can infer that the composition of pathologic and normal physiologic calcification might be similar while mechanisms of formation could be quite different. We have examined the change in crystal size with time and find that crystal volume increases up to about three weeks while the total number of crystals per unit volume decreases. It is possible that smaller crystals might re-dissolve providing additional calcium to support the continued growth of larger crystals. This process could reduce the overall surface energy as has been argued by Walton (1967).

B.B. Tomazic: The assumption that the final crystal size does not differ appreciably from the initial size may be an oversimplification, since the rate constant does depend on factors such as surface area, imperfections and defects.

Author: While this statement is quite true, we do not have enough data on the physical characteristics of these biological crystals to permit a less generalized analysis. However, if we apply this assumption only to mature crystal systems, then the validity of the model can be substantiated as is discussed in the text.