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Diffusion Systems for Evaluation of Biomineralization

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Abstract

A variety of *in vitro* study methods have been used to elucidate the roles of matrix molecules in biomineralization processes. Among these, gel diffusion-precipitation studies have proved to be an effective tool. This methodology is uniquely capable of characterizing the effects of matrix molecules on mineralization while only using very small quantities of material. Furthermore, gel methods have been extended for use as a mineralization assay system to characterize modified matrix molecules and synthetic analogues. Here we discuss the advantages and limitations of gelatin, agar, agarose, and other systems for studying the mechanisms of biomineralization.

Keywords

Hydroxyapatite; Biomineralization; Gel diffusion; Gelatin; Agarose; Agar

In Vitro Studies

The functions of vertebrate and non-vertebrate mineralized tissue matrix biomolecules, such as osteopontin [1-4], osteonectin [5,6], amelogenin [7,8], bone sialoprotein [2,3,6,9], and a variety of shell proteins [10,11] generally have been demonstrated in a three-step process. First, molecules that may influence the biomineralization process are either isolated from mineralized tissue, purified, and characterized, or cloned and expressed in recombinant forms. Their spatial and temporal distribution in mineralizing tissue, and their presence or absence from diseased or non-mineralizing tissue, as assessed by immunolocalization or *in situ* hybridization, suggests their role in mineral formation. Second, the molecule being studied can be associated with the biomineralization process *in vivo* via tissue studies on genetically modified or diseased organisms where expression of the substance of interest is altered, prevented, or enhanced. Third, *in vitro* mineralization studies may be used to provide insight into the mechanism of action of matrix proteins and to elucidate a molecule's function *in vivo* [12]. While many different techniques have been used in the *in vitro* studies, gel precipitation methods, used for over a century for the growth of large crystals [13], have recently been introduced to help elucidate how non-collagenous proteins and other biomolecules act to control biomineralization processes.

The interplay of *in vivo* and *in vitro* studies allows characterization of a molecule's role in complex living tissue, where we have limited experimental control, against a backdrop of information from well-controlled, simpler precipitation studies. The cell-free *in vitro* studies allow characterization of an individual compound or mixture's effects on nucleation, growth kinetics, inhibition, and crystal composition and habit, as well as quantitative comparisons

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among matrix molecules. Molecules under study may also be modified systematically to probe their function. For example, a protein can be phosphorylated, carboxylated, or glycosylated to varying degrees to characterize the effects of posttranslational modifications on biomineralization. We review here the recent application of crystal formation in gels to probe mineralization mechanisms with emphasis placed on hydroxyapatite formation and growth, although the techniques described are applicable to other types of biomineralization.

Most *in vitro* studies of matrix proteins involved in hydroxyapatite (HA) formation have been done in solution, as opposed to gels. The solution studies characterize the effects of matrix molecules on *de novo HA* crystal formation or crystal growth from seeds at or near supersaturated conditions. These studies monitor precipitation after mixing soluble calcium and phosphate salts in buffer, usually at physiologic temperature, pH, and ionic strength. In an important variation, pH-stat titration uses acid or base addition to maintain a constant pH without the use of a buffer while monitoring precipitation kinetics from titration data [14]. Extension of the pH-stat technique to replenish soluble calcium and phosphate ions during precipitation allows constant composition studies [15]. In the more physiologically realistic constant composition studies the driving force for precipitation does not diminish during the precipitation reaction.

Gel Precipitation Studies

The primary purpose for the adaptation of gel diffusion techniques commonly used for growing large crystals [13] to studies of the effects of matrix molecules on biomineralization was to reduce the volume of the precipitation reaction and, thus, the quantity of the biomolecule required. Non-collagenous proteins and other matrix molecules of interest are present in low amounts in tissue and thus are difficult to obtain. It is difficult, at best, to reduce the volume of a solution reaction to less than a few milliliters without the use of gels, due to constraints of stirring and evaporation. On the other hand, a biomolecule can be dissolved or suspended in a small volume of gel where it is “trapped” by diffusion [1], allowing precipitation studies to be done in 100 μL or less.

The gel study systems offer other advantages. In these studies, calcium, phosphate, or other mineral ions are slowly diffused from adjoining solutions or gels into the region of the gel containing the biomolecule under study. The physio-chemical nature of this process more realistically mimics the unique mineralized tissue matrix environment, compared to non-gel solution studies. The system allows study of *de novo* nucleation, or heterogeneous nucleation and growth, either from hydroxyapatite seeds or putative biological nucleators. This is important for elucidation of the role played by a protein in nucleation, growth, and/or or inhibition.

In the gelatin diffusion-precipitation reaction, as done in our laboratory [16], the biological agent is confined to a 100 μL volume of 10% denatured collagen gel. It is sandwiched between two 1.45-mL volumes of pH 7.4 buffered gel in a 0.79-cm inner-diameter polystyrene tube. The total “sandwich” is 3 mL in volume and 6 cm long. The system is shown schematically in Figure 1, taken with permission from J. Phys. Chem., 93, 1628 (1989). Calcium chloride (100 mM) and ammonium acid phosphate (100 mM) solutions flow past either end of the tube from “infinite” reservoirs (Ca and Pi in the figure), such that calcium and phosphate ions diffuse into the reaction volume from opposite sides. Gels are connected in parallel to the lines through which calcium and phosphate are pumped, in double diffusion tubes (shown) or closed-ended single-diffusion tubes (not shown). The reservoirs and gels are made in 150 mM Tris buffer at pH 7.4, and contain 0.010% sodium azide to prevent bacterial growth. The system is maintained in a carbon dioxide-free atmosphere to prevent carbonate incorporation into forming mineral. An air pump, regulators (A1-3) and saturated barium hydroxide solution (B) to remove carbon

dioxide are shown in the figure. More recent studies have used compressed nitrogen. The system is maintained at ambient temperature because gelatin begins to melt at 35°C. Pre-formed apatite “seed” crystals can be added along with the protein, or pre-coated with protein and then placed in the gel, enabling evaluation of effects on crystal growth in the absence of nucleation.

Double-diffusion (DD) precipitation is typically run in triplicate for a given protein concentration, in parallel with triplicate DD controls that contain no biomolecule. In addition, single-diffusion (SD) tubes that are closed at one end, and only exposed to the calcium or phosphate reservoir, are also run in triplicate, both with and without the macromolecule in the center. Thus, 18 tubes are usually run in parallel to characterize one matrix molecule at one concentration.

Calcium and phosphate concentrations at the precipitation site are significantly lower than in the reservoirs and lower than in the adjacent gel due to the diffusion gradient through the tube and the depletion of ions when the precipitate forms. The calcium-phosphate millimolar product has been shown to be 37 mM^2 immediately adjacent to the site of first precipitation in control experiments, and hydroxyapatite with a Ca/P ratio of approximately 1.5–1.7 is formed. The presence of hydroxyapatite is confirmed by X-ray diffraction (XRD) and infrared spectroscopy. Effects of macaromolecules on crystal habit and size can be monitored by electron microscopic examination of the precipitants.

The gelatin is transparent, so precipitation can be observed. Precipitation is first observed at 3 days, in the absence of matrix molecules that serve as nucleators or inhibitors (e.g., the controls). Typically, after 3.5 or 5 days, the center reaction volume is cut out and hydrolyzed for calcium and phosphate analyses. The SD tubes allow calcium or phosphate concentrations, in the absence of precipitation, to be subtracted from the DD analytical results. This corrects for the sequestering of ions by the macromolecule; typically, calcium accumulation by the polyanionic macromolecules. Promotion or inhibition of mineralization is determined by an excess or deficiency of accumulated calcium and phosphate in DD sample tubes compared to controls, after correction for SD tube analyses. Precipitates can also be isolated from the DD gels for XRD, Fourier transform infrared spectroscopy (FTIR), or other characterization methods by melting the gel followed by centrifugation.

In this system the calcium and phosphate concentrations decrease along a diffusion gradient between the reservoir and the precipitation band near the center of the gel. This design avoids shortcomings of earlier gel precipitation studies [11,17,18] that used unrealistically high concentrations of ions in or adjacent to gels to obtain acceptable deposition rates and quantities of precipitate. The diffusion coefficients, based on Fick's second-order differential equation [19], were determined for the gelatin system [16]. Because ions are replenished by diffusion from large reservoirs during the precipitation reaction, the reaction reaches a steady state crystal growth condition. Furthermore, ions that are chelated by macromolecules are replaced by subsequent diffusion.

The double-diffusion gel system, as described above, has been used extensively in our laboratories and others to probe the effects of matrix molecules on mineralization *in vitro* (Table 1). These experiments have shown *inter alia* promotion of HA formation and growth by acidic phospholipids [16], low concentrations of dentin phosphophoryn [20], biglycan [21] and bone sialoprotein [2]. On the other hand, inhibition of mineralization was observed for osteopontin [1,2], dentin sialoprotein [22], aggrecan [16], dentin matrix protein-1 (DMP-1) [23], and higher concentrations of dentin phosphophoryn [20] and dentin sialoprotein [22]. In the case of DMP-1, our recent results show that the non-phosphorylated recombinant rat protein's nucleation activity is lost upon phosphorylation [23]. This example demonstrates the gel system's use to probe the effects of chemical modifications of proteins under study. We have

also used the gel system to examine the role of matrix vesicles grown in culture to promote mineralization, extending the technique from macromolecules to organelles [24].

The concentration-dependent results for dentin phosphophoryn and other proteins that promote at low concentrations but inhibit at high concentrations may at first seem surprising. It is likely, however, that a peptide sequence that acts as an apatite template at low concentration could, at high concentrations, adsorb onto apatite surfaces and block growth. This dual-role mechanism was suggested by studies of calcium carbonate formation in invertebrates [25]. Addadi et al. [26,27] have also described conditions under which a macromolecule may promote mineralization when adsorbed on a forming nuclei or rigid substrate but act as an inhibitor in solution. Also, Hunter et al. [6] refer to unpublished results in which poly (L-glutamic acid) nucleates apatite below super-saturation conditions, but inhibits formation at higher concentrations.

Hunter and Goldberg [3] used a variation of the double diffusion gel method to explore apatite nucleation and inhibition by a number of matrix proteins. Their system uses an agarose gel separated from calcium and phosphate reservoirs by diffusion membranes, and differs from the gelatin gel system, described above, in several other particulars. They showed that perfusion of their gel with 7.5 mM calcium and phosphate solutions resulted in HA precipitation, while 6.0 mM solutions did not form precipitate. The critical concentrations of calcium and phosphate needed for precipitation in their system is therefore close to the 37 mM^2 value observed in our gelatin gel experiments. In more recent studies, Hunter and Goldberg used 6.5 mM calcium and 3.9 mM phosphate in the reservoirs, which are below the concentration threshold for spontaneous precipitation. They thus used the gel system as an assay for nucleation and growth, since inhibition could not be observed at sub-saturation conditions. Nonetheless, their gel system is similar in overall approach, employing double diffusion of ions from opposite directions into a gel that contains the matrix molecule of interest.

Hunter and Goldberg [3] observed nucleation with bone sialoprotein (≥0.089 μg/mL) and dentin phosphophoryn (≥10 μg/mL) under sub-saturation conditions. No nucleation activity was seen for the other proteins they studied in this system (osteocalcin, osteopontin, osteonectin, and chondrocalcin, all up to 100 μg/mL). The inhibition potential of these proteins was characterized in a pH-stat solution system that monitored HA formation based on the uptake of hydroxyl ions.

Interestingly, Hunter et al. [28] and Goldberg et al. [29] extended their gel diffusion method as a tool to characterize the mechanisms of protein-mineral interaction. They showed that apatite formation is induced by polyglutamic acids, requiring a sequence of eight glutamate residues [9], but not by other anionic homopolymeric peptides, supporting the hypothesis that polyglutamate sequences in matrix proteins act as nucleation sites. Furthermore, protein fractions containing polyglutamate sequences that were isolated from bone sialoprotein were shown to have nucleating capabilities [29]. Hunter and Goldberg [30] also chemically modified matrix proteins, both by dephosphorylation and by blocking of carboxylate groups, to elucidate the roles of these groups in nucleation by bone sialoprotein and inhibition by osteopontin [28]. Similarly, recent papers have used the agarose gel systems to characterize proteins made by recombinant methods or modified by site-directed mutagenesis [9,31].

Two other interesting gel diffusion precipitation systems were reported by Fujisawa et al. and Wada et al., one using electrophoretic gels and the second using a microtiter plate system. In the former, the authors used an SDS-polyacrylamide electrophoretic gel both to separate and purify proteins, and then as a calcium phosphate diffusion-precipitation media [32]. Thus the separated proteins are characterized for mineralization effects without removal from the original electrophoretic gel. Calcium and phosphate ions diffuse into the gel from opposite

sides and perpendicular to the direction of the electrophoretic separation, both by concentration gradient diffusion and by use of an electric potential. Unfortunately, opposite effects on nucleation and inhibition were observed for dentin phosphophoryns with and without an electric field. Concentrations were undefined after electrophoretic migration and their results could not be reconciled with earlier studies [33]. Note that results for phosphophoryn in our laboratory in the collagen gel-based system yielded concentration-dependent promotion and inhibition [20,34].

In comparing their results to earlier studies, Fujisawa and Wada speculate that the choice of gel can cause different results depending on the system used. This is certainly feasible, especially for gel systems that may compete for calcium binding or adsorption onto apatite nuclei or gels whose components (impurities, air bubbles, etc) may be able to act as nucleators. However many other factors could perturb the results. In particular, the proteins Fujisawa and Wada studied were separated under denaturing conditions and fixed with 10% sulfosalicylic acid, which may dramatically alter their chemistry and conformation. Additionally, the gels were dipped into phosphate solution, followed by calcium solution in the gradient diffusion experiment, so the Ca/P ratio probably varied during the precipitation process.

A second system developed by Fujisawa, et al., [35] performed gel diffusion-precipitation reactions in microtiter plates using a single diffusion system. Peptides were dissolved in agar gel containing 10 mM phosphate, and 50 μL was pipetted into each well, 100 μL of 10 mM calcium chloride was then layered over the phosphate/peptide gel. Precipitates formed in the gels upon standing at room temperature. Optical absorption was used to detect mineral deposition, based on light scattering by crystals. This offered the promise of providing a simple, rapid screening method that could assay many protein concentrations or combinations of proteins at once. The peptides studied included osteonectin, digested peptide fragments, and synthetic polypeptides (poly-glu and poly-asp sequences to mimic potential binding sites). This system was also used by Couchourel et al. [36] to study fibronectin. A single diffusion system also was used recently to characterize cooperativity between amelogenin and 32-kDa enamelin in promoting HA nucleation [37].

We have tried a modified single-diffusion microtiter method using gelatin in our laboratory because of its rapid screening potential. We encountered several difficulties, including significant swelling of the gelatin gel and subsequent uncontrolled dilution, plus back-diffusion of ions from gel into the supernatant solution. Neither of these happens in the double diffusion system. This, coupled with our concern that single diffusion methods alter protein adsorption because of the original excess of one ion (discussed further, below), led us to discontinue this approach.

Several variations on gel diffusion systems were reported by Iijima et al. [39]. In one system, the authors immersed acrylamide gel blocks containing proteins in fetal calf serum where HA precipitated after several days. The effects of percent acrylamide in the gel, CO_2 partial pressure, and the inclusion of promoters were characterized. Mineral formation was explained by the size-exclusion of high molecular weight inhibitors in serum from the gel. Mineralization was also maximized in the presence of 3.5% CO_2. Promotion of mineral formation was observed for phosvitin, phosphophoryn, demineralized dentin powder and alkaline phosphatase. Because alkaline phosphpatase is thought to cause mineral deposition by hydrolyzing organic phosphate sources, this last result suggests an inability of this system to distinguish proteins that function as nucleators and proteins with other functions.

In a second system, Iijima et al. characterized the size and shape of octacalcium phosphate (OCP) crystals formed in a 15 microliter gel composed of 10% amelogenin at pH 6.5 using a cation selective membrane and a diffusion membrane to control Ca and P entry, respectively

[8,39]. Comparisons were made to agarose, albumin, and agar gels of the same volume. This imaginative study system pushes the limit of using gels for small volume reactions. Because amelogenin comprises 95% of the extracellular protein during the formation of tooth enamel, the use of the amelogenin to form a gel rather than dissolving it in a solvent gel (as in gelatin or agarose gel studies) may approximate the *in vivo* system. OCP is believed to be a precursor to apatite in tooth enamel, as referenced by Iijima. Earlier gelatin gel studies showed that bovine amelogenins caused OCP to grow with remarkably long crystal habits [40]. Although OCP crystals formed in the amelogenin micro-gel system were smaller than in controls and had unusual crystal habits, they had elongated aspect ratios. These studies did not characterize promotion or inhibition of mineralization by amelogenins, however, Hunter et al. [7] saw no promotional effect of amelogenin in their agarose gel system, nor inhibition in their pH-stat solution system. These differences may be related to pH, as Iijima's studies were done at a low pH (6.5) where OCP tends to form, and Hunter's studies use a physiologic pH of 7.4.

He et al. [41] used a double-diffusion method to study the growth of HA on self-assembled recombinant DMP-1 that was adsorbed on glass slides. This study did not employ a gel, but calcium and phosphate were electro-diffused to the adsorbed protein site from opposite directions using an electrophoretic device. The authors noted that DMP-1 formed oligomers when incubated with $CaCl_2$. The paper characterized effects of self-organization of DMP-1 fragments and synthetic acidic polypeptides on their nucleation activity. It is interesting to consider that a gel medium (had one been used) could prevent or retard diffusion of macromolecules, thus affecting the potential for self-assembly. In a gel medium, self-assembly could be carried out before adding protein to a gel, but may not occur *in situ* in a gel. Our studies in the gelatin gel system with the same recombinant DMP-1 as described in He's paper also showed nucleation [23].

Double Diffusion and the Calcium to Phosphate Ratio

Precipitation for two-component systems under double-diffusion conditions was modeled mathematically by Pucar et al. [19]. They showed theoretically and experimentally that precipitation under these conditions followed an “equivalency rule.” Precipitation occurred from super-saturated gels with equal equivalents of cations and anions. These critical concentrations were determined for a variety of precipitation systems [42]. Critical concentrations exceeded solubility products reported previously in the literature for most systems, including calcium phosphate precipitates. Changing the ratio of cation concentration to anion concentration in the two reservoirs resulted in the precipitation band shifting position, but did not change the critical concentrations. In a later study Pokric and Pucar [43] observed critical concentrations (~4 mM calcium and 4 mM phosphate) that were similar to our later observations. However, they identified their precipitates as amorphous material with a 1:1 $Ca:PO_4$ molar ratio that later transformed to octacalcium phosphate. We have only observed HA in our system. Liesegang rings (multiple precipitation zones) [13] were formed in the Pokric study and may have contributed to differences in results.

The role of the calcium to phosphate ratio warrants special attention because this has varied widely in other approaches to gel precipitation studies in the literature (see below). Importantly, the surface charge on hydroxyapatite becomes more negative in the presence of excess phosphate and more positive in the presence of excess calcium, due to surface adsorption [44]. In addition, the adsorption of excess phosphate onto an apatite surface can displace adsorbed proteins [45]. Because the mechanisms of action by which proteins influence mineral nucleation, growth, or inhibition depend on surface adsorption, the calcium to phosphate ratio can critically perturb these processes. In studies done in solution, as opposed to gels, the calcium to phosphate concentration ratio prior to precipitation is an independent variable, controlled by the experimenter. In a double diffusion experiment, however, the calcium and

phosphate ratio in the reservoirs do not determine the critical concentrations prior to formation of the first precipitate (only the location of the precipitation band). The critical concentrations of the two diffusing ions are fixed where precipitation first occurs. This constraint does not exist in solution studies or in studies where one ion is premixed into the gel and only the second ion diffuses into the gel.

Variations Between Study Systems

In many cases there is good agreement between gel studies, for example, as in the demonstrations of the promoting effects of bone sialoprotein both in Hunter and Goldberg's work [6,9] and in our studies [2]. There are instances, however, of lack of agreement between studies which reflects the variations among study systems and protein preparations. Variations among published *in vitro* studies that could profoundly influence results include different gel materials, single vs. double (or sequential) diffusion of ions, denaturing vs. non-denaturing protein isolation or gel conditions, and variations of calcium to phosphate ratios. In addition to study system variations, there are differences in the sources and purities of macromolecules. The situation is far more confusing if one considers all studies, including solution studies as well as gels, with even greater possible sources of variation.

Few direct comparisons have been made of the effects of the type of gel used for HA formation although the literature is replete with comparisons of gel systems for growing large crystals [13]. In an early double-diffusion study by Pokric and Pucar [43] no difference was found between calcium phosphate precipitation in agar, reconstituted collagen, or denatured collagen (gelatin) gels. The conditions used in this study resulted in the first precipitated phase being amorphous calcium phosphate that transformed to octacalcium phosphate, and precipitation occurred in Liesegang rings [13]. Another early study by Hunter et al. [46] compared hydroxyapatite precipitation in collagen, gelatin, and agarose gels using a single diffusion system. They concluded that the observed differences in the rates of precipitation in their system could be explained by relative rates of ion diffusion in the different gels. Diffusion rates are determined both by gel concentration and by the structure of the gel components [13]. Although these papers did characterize the effects of different gels, finding none, they were not studies of the effects of proteins added to the gels. They could not, therefore, address the potential for protein-gel interactions. Effects of pH, individual amino acids (aspartic acid, glutamic acid, and serine), and Trizma buffer were recently characterized in a gelatin gel diffusion system [47]; pH affected the phase that precipitated while the additives affected crystal morphology.

Table 1 summarizes results from double diffusion gel studies, primarily done by Hunter and Goldberg and in our laboratory. There is clear agreement between results from these study systems for the promotion effects of bone sialoprotein and the inhibitory effects of osteopontin. There is an apparent difference in the results for dentin phosphophoryn that may reflect the concentration-dependent results. Several examples are seen in our studies of molecules that promote mineral formation at low concentrations but inhibit at high concentrations, as discussed earlier (indicated as promoter/inhibitor in the table). This effect is not seen in Hunter and Goldberg's works, where most nucleation studies were done in gels under sub-saturation conditions, where inhibition cannot be observed (They used a non-gel pH-stat solution system to probe inhibition.) The table also includes our recent results showing promotion by a non-phosphorylated, recombinant rat dentin matrix protein-1 (DMP-1), with loss of the promotional activity upon phosphorylation. Native bovine DMP-1, which is more highly phosphorylated, was shown to inhibit in the same study system, suggesting that the degree of phosphorylation controls the activity of DMP-1.

Lack of agreement among results in different studies often cannot be meaningfully analyzed because of the large number of variables involved. It is interesting to speculate, however, on the effects of interactions between biomolecules and the matrix gel material. It is likely that the function of non-collagenous matrix proteins on mineralization *in vivo* is site-specific, being influenced by the collagen matrix or other matrix proteins, or by the vesicle environment in the case of matrix vesicle nucleation. Fujisawa et al. [48], for example, provide SEM evidence that bone sialoprotein binds selectively in the gaps in collagen at sites where mineral crystals form. These interactions would not be seen in any of the gel systems unless fibrillar collagen was included. This is strongly suggestive of an interactive effect between the BSP nucleation activity and binding at this site. As pointed out by Hunter et al. [6], *in vitro* effects of a variety of proteins vary between solution studies, gel systems, and especially when proteins are bound to agarose beads or collagen. Although gel precipitation techniques were not used, Crenshaw et al. [49-51] have shown that a variety of matrix proteins that do not promote mineralization in solution, do induce mineral formation when cross-linked to agarose beads or collagen. These results suggest the need to perform side-by-side studies on individual matrix molecules with systematic variation of components in the gel matrix. Binding or lack of bonding of macromolecules under study to the gel matrix should be a critical variable. Thus, while consistent results between studies strongly support conclusions in some cases, inconsistencies between other studies maybe a clue to more complex matrix-dependent effects that are in need of systematic exploration.

Clearly, the most informative results are where there is general agreement among studies in different systems. In addition, consistent results from one system that is used to probe molecular structure-function relationships, as in the Hunter and Goldberg example, are convincing in their self-consistency. In any event, *in vitro* studies can, at best, support hypotheses about *in vivo* function. Substantiation of *in vitro* results by *in vivo* studies is the most significant confirmation of conclusions.

Conclusions

We believe that double-diffusion gel precipitation systems are the most realistic and practical of the cell-free *in vitro* methods of studying biomineralization processes. They can allow study of precipitation kinetics, if multiple time points are analyzed, and characterization of the resulting mineral under realistic conditions of immobilized matrix molecules at near-physiologic calcium and phosphate saturation conditions. These methods provide a portrait of how matrix molecules act to nucleate, inhibit, or modify mineral formation, and how the structural features of these molecules affect the mineralization process.

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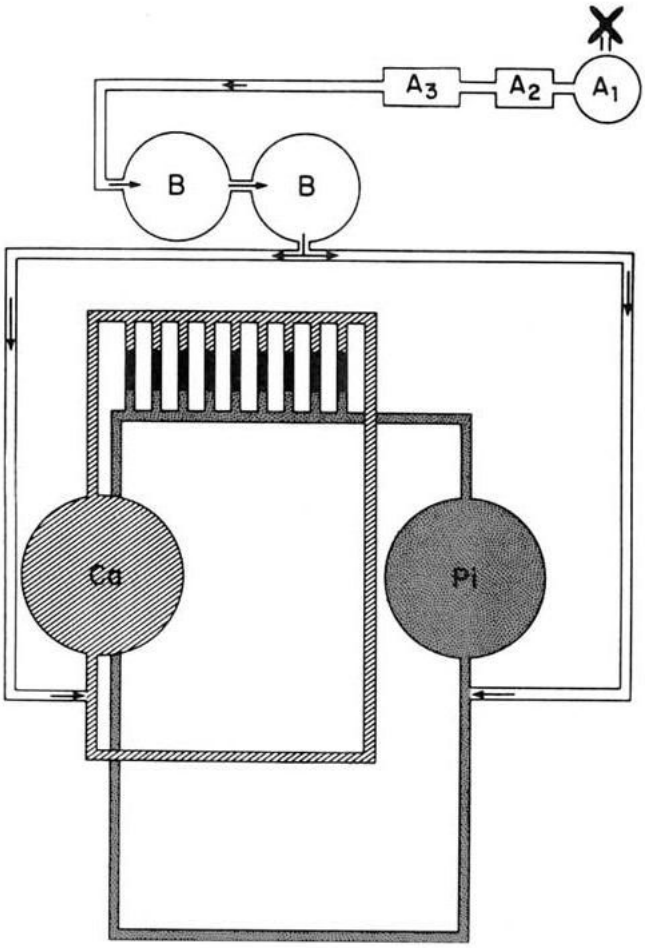

Fig. 1.
Schematic diagram of the dynamic hydroxyapatite growth formation system. Reprinted from J. Phys Chem, 1989 [16] with permission.

Table 1

Gel diffusion studies of effects of matrix molecules on HA formation

Molecule	Source	Gel	Effect	micro-g/mL	Reference
Acid phospholipids	Bone	Gelatin	Promoter	0.3–1.2	16
Aggrecan	Cartilage	Gelatin	Inhibitor	2	16
Albumin	Serum	Agarose	No effect	3–30	3
Ameloginins	Teeth	Gelatin	Longer Habit**	1250–20000	40
Ameloginins	r-murine	Agarose	No effect	1–300	7
Ameloginins	r-murine	Amelogin Gelatin***	Longer Habit**	10000	8
Ameloginin and enamelin	Teeth		Promoter	7500–15000/18-80	37
Bone sialoprotein dephosphorylated	Bone	Agarose	Promoter	5	28
Bone sialoprotein	Bone	Agarose	Promoter	0.3-10	6
Bone sialoprotein	Bone	Agarose	Promoter	0.087–0.87	9
Bone sialoprotein	Recombinant	Agarose	Promoter	1.7-3.4	9
Bone sialoprotein	Bone	Gelatin	Promoter	10-50	2
Bone sialoprotein	Bone	Agarose	Promoter	1	1
Chondrocalcin	Cartilage	Agarose	No effect	0.3-100	6
Collagen I	Tendon	Gelatin	No effect	1000	16
Collagen I	Skin	Acrylamide	No effect	1000	38
Collagen X	Calcified cartilage	Gelatin	No effect	50-300	34
DS-biglycan	Cartilage, bone	Gelatin	Promoter/Inhibitor	10-50	6
DS-decorin	Skin	Gelatin	No effect	1-100	21
Dentin sialoprotein	Dentin	Gelatin	Promoter/Inhibitor	5-10 / Inn 50-100	22
Dentin matrix protein-1	r-DMP-1 (rat)	Gelatin	Promoter	1-25	23
Dentin matrix Protein-1	r-DMP-1 (rat) +PO4	Gelatin	No effect	0.5-28	23
Dentin matrix protein-1	b-DMP-1 (bovine)	Gelatin	Inhibitor	5-25	23
Osteopontin	Bone	Agarose	Inhibitor	100	28
Osteopontin	Bone	Agarose	No Effect	1-10	3
Osteopontin	Bone	Gelatin	Inhibitor	10-100	2
Osteopontin	Bone	Gelatin	Inhibitor	25-100	1
Osteopontin	Bone	Agarose	No effect*	0.3-100	6
Osteopontin	Bone	Agarose	No effect*	0.3-100	6
Phosphophoryn (+ 1 mM Ca)	Dentin	Gelatin	Promoter/Inhibitor	0.01-1 / 10-100	20
Phosphoryn	Dentin	Agarose	Promoter	10-30	6
Phosphoryn	Dentin	Acrylamide	Promoter	1000	38
Phosvitin	Egg Yolk	Acrylamide	Promoter	1000	38

* Inhibited in parallel solution study (not gel)

** OCP formed pH 8, promotion or inhibition not characterized

*** Single-diffusion study