Published in final edited form as:

CrystEngComm. 2012 January 1; 14(18): 5681–5700. doi:10.1039/C2CE25289A.

Rediscovering Hydrogel-Based Double-Diffusion Systems for Studying Biomineralization

Jason R. Dorvee¹, Adele L. Boskey², and Lara A. Estroff^{*,1}

¹Department of Material Science & Engineering, Cornell University, Ithaca, NY 14853

²Weill Medical College, Cornell University and The Hospital for Special Surgery, New York, NY 10021

Abstract

For those seeking to model biomineralization *in vitro*, hydrogels can serve as excellent models of the extracellular matrix (ECM) microenvironment. A major challenge posed in implementing such systems is the logistics involved, from fundamental engineering to experimental design. For the study of calcium phosphate (*e.g.*, hydroxyapatite) formation, many researchers use hydrogel-based double-diffusion systems (DDSs). The various designs of these DDSs are seemingly as unique as their applications. In this Highlight, we present a survey of four distinct types of double-diffusion systems and evaluate them in the context of fundamental diffusion theory. Based upon this analysis, we present the design and evaluation of an optimized system. The techniques and framework for the evaluation and construction of a DDS presented here can be applied to any DDS that a researcher may want to implement for their particular studies of biomineralization.

1. Introduction

A challenge in the field of biomineralization is identifying the appropriate *in vitro* model for studying a particular mineralization phenomenon. Depending on what aspects of the process one wants to model, the type of *in vitro* system can vary greatly: from solution growth of hydroxyapatite (HA) using a constant composition set-up,^{1, 2} to *in vitro* mineralization using cells,³ to growth in hydrogels.^{4–7} Traditionally, crystal growth in hydrogels is a technique used by crystallographers to grow large crystals^{8, 9} and to study how those crystals grow within a hydrogel matrix, sometimes as a function of time.¹⁰ For those seeking to model biomineralization, hydrogels are excellent models of the extracellular matrix (ECM) microenvironment. For example, when proteins are incorporated into gel-based assays, their function can more closely mimic the behavior found in a biological system, as compared to more standard solution-based assays.⁶ In one example, the introduction of recombinant amelogenin, a protein found in immature enamel, into a hydrogel matrix led to the growth of high-aspect ratio octacalcium phosphate (OCP) crystals, which were elongated in the direction of ion flow.^{11, 12} In other reports, the integration of osteopontin (OPN) into a gelatin hydrogel revealed that both the concentration of OPN and the degree of phosphorylation of OPN, affected the protein's role as either an inhibitor or a promoter of mineralization.^{6, 13, 14} Finally, the mineralization activity of bone-sialoprotein (BSP) was compared with that of a modified BSP, which lacked the collagen binding domain.¹⁵ The unmodified BSP was a stronger promoter of hydroxyapatite (HA) formation in a collagen hydrogel, suggesting that the binding of BSP to collagen contributed to its function in mineralization. All of these examples demonstrate the potential of hydrogel-based systems for developing *in vitro* models of the biomineralization processes in bone and teeth.

*lae37@cornell.edu.

We have recently sought to expand the diversity of experiments that can be performed using double-diffusion (also called counter-diffusion) systems (DDSs) to study the mineralization in an ECM-like environment.¹⁶ In our explorations we quickly discovered, as with most *in vitro* models for mineralization, the challenge and complexity¹⁷ in creating and building such a system. We also discovered the difficulty in trying to use these types of systems for applications beyond their original purpose. It is this very issue that has motivated this “Highlight” article and the experimental results presented herein.

2. Types of Hydrogel-Based Double-diffusion Systems

The main components of any hydrogel-based DDS are a hydrogel and opposing sources (reservoirs) of ions on either side of the gel. The hydrogel becomes the medium through which ions diffuse (creating an ion gradient), and in which mineralization takes place (Figure 1). Several different hydrogel-based DDSs have been described in the literature for studying the formation of calcium phosphate minerals,^{6, 10, 18–29} and their results have been reviewed elsewhere.^{6, 7} It is difficult, however, to compare the performances of these systems because of a lack of information in the literature with regards to the design specifications of each system, and how or why these DDSs and their applications differ from each other. Furthermore, not all designs detail the relationships among the specific components of each system and the corresponding diffusion theory or why those components were chosen for a given experimental application. In addition, there are unexplained variations in the reaction times for each system, as well as how each system is used in its application.⁶ Here, we demonstrate that when four representative systems are examined in the context of fundamental diffusion theory,^{30–33} even though they differ in their specific application, there is no difference in the techniques required to evaluate them. By identifying and understanding the diffusion theory that is common to all systems, we can now compare and evaluate these systems across the different designs, applications, and fundamental research objectives. In addition to analyzing examples from the literature, we conclude this “Highlight” by presenting a series of evaluation strategies for the design and testing of DDSs for future studies in biomineralization.

In this work we focus on passive DDSs, *i.e.*, systems that rely on differences in chemical potential for diffusion. Passive DDSs can be separated into two categories: static and dynamic (Table 1). The physical details of a system (*e.g.*, the path length, the capacity of the reservoirs, the reactant concentrations) all contribute to defining the boundary conditions (Table 1). Differences in boundary conditions and system variables change how a particular diffusion system is evaluated (mathematically) and how a diffusion system is used (experimentally). The primary variable between static and dynamic systems is the length of the gel and its relation to the timescale of the experiment. It should be noted that any of these passive systems can be modified to perform as active diffusion systems with the introduction of external forces such as an electric field.^{34–38} As we go through each example from the literature, we will describe the mathematical treatment, construction, and respective advantages and disadvantages of each system for a range of applications.

2.1 Static Systems

Static diffusion systems, such as those employed by Iijima and Oldak^{19, 39–42} and Hunter and Goldberg,^{4, 5, 19, 39–42} rely on the hydrogel being immediately brought to equilibrium (from the start of, or early in the timeframe of the experiment) with the ion(s) kept in reserve in the reservoirs (Fig. 1a). In other words, the flux of ions into and out of the gel reaches a steady or static state.^{19, 20, 33, 39–42} One of the keys to creating a static DDS is restricting the length of the gel to a size such that the time it takes to reach a steady-state condition, with respect to ion diffusion, is reached relatively quickly with respect to the overall time frame of the experiment. The defining moment when steady-state is achieved occurs when the

relaxation time τ (in seconds) is much greater than the length of the sample L squared (in centimeters) divided by the minimum diffusivity (Eq. 1).^{33, 43}

$$\tau \gg L^2/D_{\min} \quad (\text{Eq. 1})$$

Since the value of D for small ions in gels is typically on the order of $10^{-5} - 10^{-6} \text{ cm}^2/\text{s}$,^{4-6, 13, 14, 18, 19, 40-42, 44-49} only L can be varied such that the time τ required to reach steady-state becomes very small.

The governing law for the movement of materials in a static diffusion system is Fick's 1st Law (Eq. 2); time is not a factor because steady-state is reached rapidly. As such, the flux, j , is dependent simply on the diffusivity, D , of the diffusing species and the change in concentration, c , over the change in distance from the source, x (Fig. 1a and Eq. 2).

$$j_1 = -D_1 \frac{\partial c_1}{\partial x} \quad (\text{Eq. 2})$$

Accordingly, the value of c only changes within the hydrogel with distance from the source, not with time (Fig. 1a). The steepness of the resulting linear concentration gradient is dependent on the total length of the hydrogel from x_0 to L . The longer the gel is, the more gradual the concentration gradient. In contrast, the shorter the gel is, the steeper the resulting concentration gradient.

There are cases of static DDSs where the path length of the gel is so small (or the gel is replaced by a membrane) that there is essentially no flux through the gel, because the concentration of ions in the gel has reached the same concentration of ions in the reservoir.^{27, 50-52} If x is sufficiently small (and D of an ion within the hydrogel is smaller than D of an ion outside the hydrogel) the system reaches equilibrium quickly and the flux of reactant, j , throughout the length of the gel from x_0 to L becomes zero. In cases where the source concentration of the diffusing species is exceptionally high and the length of the gel is exceptionally small:

- flux into the gel is zero (flux j from $x < x_0$ to $x = x_0$ is zero)
- flux through the gel is zero (flux j from $x = x_0$ to $x = L$ is zero)
- flux out of the gel is high (flux j from $x = L$ to $x > L$ is not zero).

In this sense the small value of L for the system allows for the gel to be treated as a membrane.³²

Precipitation in a static DDS can occur over a range of supersaturation levels, depending on how the system is designed and whether nucleation or growth is being studied. A sub-threshold level⁵ is when the supersaturation, with respect to precipitation in the gel, is so low that it can be used to study heterogeneous nucleation and growth. A sub-threshold level is also sometimes described as a pre-nucleation supersaturation, where homogeneous (spontaneous) nucleation is not the governing nucleation mechanism and only mineralization promoters can trigger nucleation. In contrast, a super-threshold level⁵ is achieved when the supersaturation with respect to precipitation in the gel is high enough that it can be used to study the growth of crystals by providing a system where they are guaranteed to nucleate and grow, without the need for seed crystals or mineralization promoters. Since at a super-threshold level nucleation always occurs, only the degree of mineralization and the effects of additives on growth modification can be observed under this condition.

2.1.1 The Dual-Membrane Nested Tube Static DDS—The Dual-Membrane Nested Tube static DDS (Fig. 2) was designed to study the effects of ionic flow on crystal growth.^{12, 53, 54} By using a cation selective membrane on one side of the gel chamber, directional ion flow is obtained. In the absence of any additives, elongated OCP crystals form with their long-axis parallel to the direction of the ionic flow.¹¹ The same system was later adopted to study the effects of small quantities of proteins and gels on crystal growth.^{11, 12, 20, 55–59} For example, the system was used to study the effects of recombinant amelogenins on the crystal morphology of OCP. Amelogenins were found to limit the growth in the *ab* directions, leading to rod-like, as compared to plate-like, OCP crystals. It is important to note that the final crystal morphology is a result of both the membrane-directed ion flow, which gives elongated crystals, and the hydrogels or the proteins, which are responsible for modifications of the relative dimensions of the crystals.

From an engineering perspective, the design of the Dual-Membrane Nested Tube static DDS is relatively straightforward. At its core, the design is simply a tube placed inside a larger tube with an end of the smaller tube capped by a gel encased between two different membranes. The membranes are held in place with silicone O-rings and a plastic cap that locks the membranes in place. The larger tube is filled with phosphate and the smaller tube is filled with calcium. The specific system used by Iijima and Oldak^{11, 54, 55, 57–59} to study amelogenin gels is more intricate and includes a dialysis membrane (phosphate side), a cation-selective membrane (calcium side), a jacket on the larger tube to maintain a specific temperature, a stir bar in the larger tube (120 mL bottle) to create turbulent mixing, and a floating, smaller tube (2 mL bottle) inside the larger tube, much like a dialysis cassette in a large beaker. Some important points to consider that may affect mineralization studies in this system:

1. Since the system operates at super-threshold conditions, mineral formation is not confined to the gel. Mineral can and does form on the exterior of the membranes.
2. As a result of the design one or both of the ion reservoirs must be considered finite, creating an “infinite” reservoir source with this design is difficult to achieve (see Section 3).
3. Since the gel is only 15 μ L in volume:
 - i. Only a small amount of mineral is available for study.
 - ii. The resulting path length (300–750 microns) is so small that it is difficult to discern visually if precipitation has occurred.
4. The reaction conditions (pH and reactant concentrations/ratios) used by Iijima and Oldak favored the formation of octacalcium phosphate rather than HA.

2.1.2 The Flowing Infinite Reservoir Static DDS—The Flowing Infinite Reservoir Static DDS (Fig. 3), is a modified version of the design by De Jong et al.⁶⁰ where the acrylamide film (used in the original design) is replaced by a hydrogel. Since the diffusing solutions are maintained at constant composition by using a flow-through, rather than a circulating reservoir design, this type of DDS is most closely related to the constant composition systems used for solution-based mineralization studies.^{1, 2, 27} This type of system is used to determine whether or not particular extracellular matrix molecules promote or inhibit mineralization in various kinds of hydrogels.^{19, 39–42} For example, utilizing two different hydrogels (agarose and collagen) with similar ion diffusivities⁵ and similar level of stability at 37°C, Hunter and Goldberg examined the contribution of binding BSP to collagen on the ability of BSP to promote mineralization.¹⁵ Placing modified BSP without the collagen binding domain in both collagen and agarose produced the same effect,

while unmodified BSP (with collagen binding domain) showed a greater ability to mineralize in collagen over agarose.

From a fabrication standpoint, the Flowing Infinite Reservoir static design is straightforward to assemble. A delivery chamber is fastened to either side of a dialysis cassette and a peristaltic pump is attached to each delivery chamber. The ions (calcium or phosphate) flow into each delivery chamber at a rate slow enough to fill the chamber so that there is a continuous solution/membrane interface but fast enough that the solution is not depleted of ions before it is replaced by new solution. These assemblies are small enough that they can be placed in an incubator, which makes it possible to regulate the temperature. The design used by Hunter and Goldberg is a custom-fabricated mold for the gel, made from a machinable plastic that is chemically modified with silanes to assure good gel adhesion to the chamber and then capped at either end with a dialysis membrane before the solution delivery chambers are attached.⁶¹

Some important points to consider that may affect mineralization studies in this system:

1. The gel is cast between dialysis membranes to insure that the proteins placed in the hydrogel do not diffuse out of the gel.
2. This system has been designed to primarily operate at sub-threshold conditions. The system can be used to determine whether or not an additive promotes HA formation in a hydrogel, but it is difficult to determine whether or not an additive inhibits HA formation.
3. This system can be operated at super-threshold conditions to elucidate the degree of mineralization induced by additives. Under these conditions, however, it would be difficult to determine whether or not those additives are responsible for triggering the mineralization event.

2.2 Dynamic Systems

Dynamic double-diffusion systems (see Figure 1b and Table 1), such as those used by Boskey^{3, 13, 14, 18, 44–49} and Mandel,^{10, 62–67} have long sample path lengths, L , and reservoirs that act as sources of reactants for the hydrogel.¹⁸ In dynamic systems, the path length, L , of the hydrogel is sufficiently large that steady-state is not reached within the time frame t of the experiment. Concentration gradients of the reactants within the hydrogel are dynamic (flux of reactants in and out of the hydrogel is not constant) and the concentrations of reactants in the hydrogel are less than the initial concentrations in the reservoirs. Since the flux of material is itself variable, the governing law for the movement of material is Fick's 2nd Law (the derivative of Fick's 1st Law (Eq. 2) with respect to x), where the flux, j , is itself changing at a given point x during t_1 to t_2 (Fig. 1b and Eq. 3).

$$\begin{aligned}
 j_{i(1)} &= -D_i \frac{\partial c_{i(1)}}{\partial x} \neq j_{i(2)} \\
 j_{i(2)} &= -D_i \frac{\partial c_{i(2)}}{\partial x} \neq j_{i(1)} \\
 j_{i(1)} - j_{i(2)} &= \frac{\partial c_{i(1)}}{\partial x} - \frac{\partial c_{i(2)}}{\partial x} = \Delta x \frac{\partial c_i}{\partial t} \\
 \Delta x \frac{\partial c_i}{\partial t} &= \frac{\partial j_i}{\partial x} = \frac{\partial}{\partial x} D_i \frac{\partial c_i}{\partial x} \\
 \frac{\partial c_i}{\partial t} &= D_i \frac{\partial^2 c_i}{\partial x^2}
 \end{aligned} \tag{Eq. 3}$$

A dynamic DDS operates across the supersaturation threshold window (from sub-threshold to super-threshold) because the ion product at a particular position x evolves over the course of the experiment. In both static and dynamic systems, precipitation events in the gel are used as 'self-checks' to assure that the system is working properly. Since the precipitation

event in a dynamic DDS cannot be set to ‘on’ or ‘off’ at the start of the experiment, like a steady-state (static) system, the ability to predict or even program the timing of such an event in a dynamic DDS is important. When and where these precipitation events appear within the hydrogel can be programmed into the design of the system (using both the initial conditions and the boundary conditions).

2.2.1 Thin Film Source Dynamic DDS—A Thin Film Source Dynamic DDS,^{10, 62–67} as used by Mandel, is the easiest dynamic design to fabricate (Fig. 4) and was adapted from a classical method for growing crystals for crystallography.^{8, 68, 69} Layers of gel with and without ions are cast into a test-tube. The layers at either end serve as thin film sources for the ions, with an ion-free layer in the middle dictating the path length. This design allows for modular changes to the system, where sections of gel can be added or substituted with various ions, molecules, or even different pH values. Once set, the system is left to drift towards equilibrium. The precipitation reactions take place over the course of several days to months. With this type of dynamic DDS, Mandel was able to produce a map of various polymorphs of calcium pyrophosphate as a function of both time and position within the hydrogel.^{10, 66}

Some important points that should be considered when using this type of system:

1. The system originally used gelatin as the principle hydrogel, but the uncomplicated design allows for the substitution of various kinds of hydrogels.
 - a. Commercially available gelatin comes from mixed batches of denatured collagen (see Section 3.2)
 - b. Through extensive study, Mandel chose of a type A 275 Bloom gelatin for its optical clarity and well-characterized composition.
2. This type of experiment uses thin layers of ion-doped gel acting as “thin-film” finite reservoir sources, and as a result, the value of c_0 in those thin films diminishes over time, thus reducing the rate of ion diffusion over time, and leading to long experimental times (weeks to months).
3. The fundamental design of the system allows it to be setup and left alone, without the need to monitor reservoirs, pumps, etc.
4. The original system was designed to operate between 5–25°C but these experiments could be done at higher temperatures if a gel other than gelatin was used.

2.2.2 Circulating Semi-Infinite Reservoir Dynamic DDS—The Circulating Semi-Infinite Reservoir Dynamic DDS as used by Boskey,^{6, 13, 14, 18, 44–49} is designed to investigate the role of small amounts of non-collagenous proteins on the initiation of mineralization as well as their effect on seeded crystal growth in mineralization within a gelatin hydrogel (Fig. 5). The system has also been carefully designed and tested to predictably and reproducibly grow HA crystals, rather than any of the other calcium phosphate phases often encountered in such systems. In this system the hydrogel matrix not only serves as a mineralizing environment, but also as a means to regulate ion delivery via diffusion, with the mineralization space making up only 5% of the total volume of the gel. Taking advantage of the ability to target the location of mineralization, this system has been used to examine the effects on mineralization of various biomacromolecules such as: lipids, matrix vesicles,^{6, 14, 45, 46} proteoglycans,⁴⁹ collagen fibrils,¹⁸ OPN,^{13, 14} and other noncollagenous proteins.^{13, 44, 47}

Of all the systems presented in this paper, this one is the most complicated from the standpoint of both engineering and fabrication. Essentially the design of the Circulating Semi-Infinite Reservoir Dynamic DDS can be viewed as multiple Flowing Infinite Reservoir Static DDSs stacked in parallel, with longer gels and a circulating, rather than flow-through, reservoir configuration. Two large vessels, 3–4 L (glass bottles with hose barbs), are constantly circulated with compressed air or an air pump piped into the fluid circuit, with the bubbles taking the shortest path to flow into the reservoirs and venting out through the vessels. The movement of each discrete air-bubble packet pulls fluid along a short path and draws the solution through the circuit into the reservoir vessels creating a turbulent, semi-infinite reservoir system, while at the same time scrubbing CO₂ out of the solution. The original system was designed to hold 18 gels for a single set of experiments connected to the reservoirs through a circuit of flexible tubing. The casting of the gels, is perhaps the most user-friendly part of the design. The layered gel strategy as used by Mandel (Fig. 4) is employed to make gels loaded with proteins at specified locations for mineralization. The gelatin solution is loaded into tubes cut from plastic pipettes which provide built-in graduation marks that aid in the mapping of both ion and mineral gradients. The tubes are filled with one end capped by a small, removable, plastic disc and parafilm. Once the gels are cast, the parafilm caps are removed from the tubes and the tubes can be placed on the diffusion system. Some important points that should be considered when using this type of system:

1. As is expected with a dynamic DDS, the onset of the mineralization process is longer than a static system, with mineralization starting around 3 days and considered “complete” (the mineral band is opaque and clearly visible) after 5 days.
2. The system uses gelatin as the principle hydrogel and that carries with it the same issues as the system used by Mandel.
3. Unlike the layered system as used by Mandel, the large circulating reservoirs provide a nearly inexhaustible source of ions (on the time scale of the experiment).
4. Even though relatively large amounts of mineral are produced compared to static systems, the mineral density per volume of gel is still low (~3% dry weight after a 5 day experiment).

2.3 Other Geometries

The representative designs presented here for static and dynamic diffusion systems are specific to the researchers who have used them. Other geometries such as commercially available U-tubes^{8, 28, 70} and gel accupuncture methods (*e.g.*, the Granada Crystallization Box, GCB)^{71, 72} can be integrated into whatever DDS design you choose to pursue. Important considerations that should not be overlooked when choosing an experimental set-up include the effects of size/scaling⁹ and geometry of the tube.²⁸ For example, researchers have reported differences in crystal morphologies in octacalcium phosphate crystals grown in U-tubes vs. straight test tubes when using agar gels in glass tubes.²⁸ Detailed exploration into these factors, however, is outside the scope of this highlight.

3. Types of Hydrogels

The hydrogel is a key component to each of the DDSs presented here. Knowing the chemical and physical properties each type of hydrogel yields insight into how and why a particular hydrogel is chosen to be paired with a particular DDS. A number of synthetic and natural polymers can be made into hydrogels,⁷³ but three types of physical gels have been primarily used for the study of calcium phosphates: agarose,^{6, 19, 21, 42} gelatin,^{6, 13, 18, 21, 40, 44–49} and collagen.^{6, 15, 19, 21, 42}

3.1 Agarose

Agarose is a polysaccharide purified from seaweed that can form hydrogels. Types of agarose are categorized by purity, strength, chemical modification,⁷⁴ and melting temperature. Both the thermal stability and gel strength are closely tied to the relative entanglement of polymer chains within the hydrogel and the hydrogen-bonding between those chains. With increasing gel strength the maximum melting temperature also increases, although the thermal hysteresis of different types of agarose (between melting and setting temperature) can vary widely and should be checked before purchase and use. A few important points about agarose that may contribute to its use in a DDS are:

1. The minimum concentration of agarose to create a hydrogel is ~ 0.1 w/v %.
2. The gelling temperature varies depending on the type of agarose and can be as low as 25 °C and as high as 70 °C.
3. The hydrophilicity and strong self-hydrogen bonding character of agarose often makes it difficult for agarose to strongly adhere to the inside of a container.
4. With decreasing gel strength, agarose bonds less with itself and is therefore more “sticky” in character.
5. Agarose gels are often brittle and therefore certain levels of mechanical stress (when handling) can lead to permanent cracks or fracture of the gel.
6. Below their gelling temperature, agarose hydrogels tend to shrink in water.

3.2 Gelatin

Gelatin hydrogels are protein-based gels made of blends of various purified lots of denatured collagen. Due to large batch-to-batch variations in purity and properties, it is recommended that a single batch of gelatin be used for all studies.⁷⁵ Acid-purified gelatins (Type A) have isoelectric points of pH 6.5–9, whereas alkaline-purified gelatins (Type B) have isoelectric points of pH 4.8–6.⁷⁶ As such, it is important to consider the type of gelatin and the pH at which it is used. In addition to the purification method, there is also variation in gelatin derived from different organisms and locations from which it is harvested from within those organisms. A “best practices” option when choosing a gelatin, is to use a gelatin type and manufacture known to be of a certain level of purity.^{62, 64, 65} Another way to obtain high-quality gelatin is to prepare it in-lab from collagen sources.⁷⁶ A few additional points about gelatin that may contribute to its use in a DDS are:

1. The minimum concentration of gelatin to create a hydrogel is ~5 w/v %.
2. The maximum temperature at which gelatin will remain as a hydrogel is ~27°C.
3. Continuous and/or repetitive heating and cooling of gelatin solutions can lead to an increase in the local ordering within the hydrogel⁷⁶ that may influence the crystallization results.
4. Gelatin is hydrophobic and has a tendency to be both adhesive and cohesive, leading to a gel that adheres well to the vessel in which it is contained.
5. Below their gelling temperature, gelatin hydrogels tend to swell in water.

3.3 Collagen

Collagen hydrogels are protein-based gels that take advantage of collagen’s tendency to self-assemble. Typical collagen gels are based on collagen type-I, purified from rat tail tendons,⁴⁰ but the sources and types of collagen used can vary based upon the user’s preferences. A collagen gel is formed by raising the pH of an acidified collagen solution to a

value of around 7, this change in pH allows for collagen fibril formation and subsequent self-assembly. Collagen gels have the advantage over gelatin gels in that they are stable at 37°C and “cleaner”.^{77, 78} A few important points about collagen gels that may contribute to its use in a DDS are:

1. The minimum concentration of collagen to create a hydrogel is ~ 0.1 w/v%.
2. Upon setting, collagen gels have a tendency to shrink.
3. Collagen can be expensive if purchasing already prepared solutions. If you purify your own collagen, the preparation can be more involved compared to using gelatin or agarose.

3.4 Effects of the physical properties of the hydrogel

In addition to the theoretical considerations for designing and operating a DDS (Section 5), the practical aspects, such as the choice of hydrogel can also have important consequences. Understanding both the chemical and the physical properties of each of these gels can guide your choice of hydrogel for your DDS and your mineralization study.

The shrinking or swelling of the hydrogel matrix changes the boundary conditions (t_0 and x_0) of the sample/experiment, and therefore changes the effective/calculated diffusivity of the solute (Fig. 6). It is important to understand that this phenomenon has both a thermodynamic and kinetic component. Thermodynamics dictate the enthalpy of mixing between the hydrogel and the diffusing solvent, and the kinetics of the system describe the diffusion of both the solvent and solutes into the hydrogel.

Thermodynamic concepts for describing solvent/polymer interactions (the χ parameter) can be applied to the solvent/hydrogel interaction. With increasing χ there is a resulting increase in the amount of solvent (water) in the volume fraction of the hydrogel (swelling). With decreasing χ there is a resulting decrease in the amount of solvent (water) in the volume fraction of the hydrogel (shrinking). In thermosetting gels such as gelatin and agarose, the polymer-solvent relation has a baseline qualitative χ parameter that can be termed either good, poor, or theta.⁷⁹

- A “good” solvent will swell a polymer/hydrogel network, physically increasing the size of the network, making $x_0 < 0$.
- A “poor” solvent will shrink a polymer network, physically decreasing the size of the network, making $x_0 > 0$.
- A “theta” solvent will neither swell nor shrink a polymer network therefore the value of $x_0 = 0$.

For example, gelatin, which has strong polymer-water interactions, swells in water below its gel temperature, whereas agarose, which has strong polymer-polymer interactions, shrinks in water below its gel temperature. As a result gels cast into diffusion tubes without an interface barrier, such as a dialysis membrane,^{12, 19, 20, 41, 58} will have at least one (if it is a single-diffusion tube) unconfined end that will swell or shrink. If the interface between gel and solution swells or shrinks it will change the overall path-length of the tube L and by default the location of the starting point x_0 (Fig. 6).

The kinetics of solvent and solute diffusion in the hydrogels can also effect t_0 and x_0 . Diffusion of solvents in many polymers cannot be described based on the concentration-dependent form of Fick’s Law with constant boundary conditions.³¹ For example structural changes due to solvent-based swelling or shrinking are strongly time dependent in glassy polymers. On the other hand, rubbery polymers can respond quickly to environmental

changes such as temperature and solvent. Hydrogels are different than both rubbery and glassy polymers. One key difference as compared to glassy polymers is that the open network of the hydrogel does not need to change to allow diffusion. However, a change in the hydrogel structure does change the values of x and t in the diffusion equation (Fig. 6). Another difference is that since hydrogels are networks of polymers that are structured by water, the diffusion of water is decreased due to hydrogen-bonding interactions with both itself and the polymer.³² The relative strengths of polymer-polymer and polymer-water interactions play a large role in determining the physical properties of the gel,³² in particular the degree of swelling/shrinking in water and the ionic diffusion rates. A solute diffusing through a hydrogel on the other hand, has limited interaction with the polymer component due to the dispersive nature of the network, as a result swelling or shrinking in a radially confined hydrogel (such as in a tube) has an effect on the value of x and t , but little, to no, effect on the diffusion of the solute itself within the tube. In situations where solute diffusion is intimately tied to the diffusion of the solvent in which they reside, factors that effect solvent diffusion will have an effect on solute diffusivity. For example the diffusivity of calcium chloride has been reported to vary between hydrogels.⁴¹ In section 5.2.1, the roles of all of these properties of hydrogels will be described in the context of the design and operation of a DDS.

4. The conditions of infinite and semi-infinite

Practical consideration of the components of a DDS involve not only the chemical/physical properties of the materials such as hydrogels (Section 3) but also the dimension and scaling of the experimental setup. An important concept for defining boundary conditions in any of these DDSs is “infinite” and “semi-infinite”. In mathematics, an infinite object or sample has no beginning and no end; it extends from $-\infty$ to ∞ .³¹ An ion diffusing through an infinite sample would start from a point source located at some position x within the sample and then (in the case of a one dimensional sample) diffuse in two directions, $-\infty$ and ∞ .^{31–33, 43} A semi-infinite object has a beginning, x_0 , but no end. An ion diffusing through a semi-infinite sample would start at some start point x_0 and (in the case of a one dimensional sample) continue to diffuse towards ∞ .^{31–33, 43}

Practically, however, an object is never truly infinite or semi-infinite. The point when or where an ion ceases to continue to infinity needs to be defined. According to Shewmon,³³ a sample has an infinite end if:

$$L > \sim 4.6 (Dt)^{1/2} \quad (\text{Eq. 4})^1$$

As a solute diffuses through a material, if the experiment is ended before the solute reaches the end, or it diffuses past the end into oblivion (lost),³³ or high dilution ($0.1\% c_0$), then the sample (in this case the gel) is defined as having an infinite end. If the solute reaches the end of the material and is reflected back into the sample, then concentration will build up over time (in which case a trigonometric series solution is required). These definitions will become important in our discussion of establishing and defining boundary conditions for DDSs (see Section 5).

5. Evaluation and Design Strategies for DDSs

Given the diversity of DDSs to choose from, here we present a set of evaluation and design strategies for the reliable and reproducible use of hydrogel-based DDSs for specific

¹The actual value for the coefficient is in the range 4.6 – 4.8.

crystallization studies. To begin the selection of a DDS for a particular type of study, the first step is to identify the main goals for the study at hand. Based upon these goals and the target questions of the study, the appropriate type of DDS (*e.g.*, static or dynamic) can be chosen (Table 2). A researcher can either choose to evaluate and modify an existing DDS, or if necessary, design a new DDS to satisfy their needs. Once constructed, any system can be tested using experiments to probe the effect the design has on the theoretically and empirically defined boundary conditions.

In the evaluation of an already established DDS, the origin of the solution to the diffusion equation must first be understood. In order to obtain a solution, the first step is to determine what kind of diffusion system it is (static or dynamic) based on the path length L of the gel, the time frame of the experiment, and applications for which the system has been used (Tables 1 and 2). The next step is to determine what boundary conditions and variables are dependent on or independent of the system design. Finally, the corresponding solution to the diffusion equation to which those variables apply can be determined. This solution can be directly obtained by solving Fick's Laws from first principles or by simply looking up an established solution in the literature.^{31–33, 43} Based on the solution to the appropriate diffusion equation, the key boundary conditions and variables are determined and accounted for in the design of the DDS.

5.1 Solving the diffusion equation

We have described two types of diffusion systems, static and dynamic. To understand fundamentally how these systems work, and to design a system of our own, we need to solve the diffusion equation. The variables that determine the corresponding solution to the diffusion equation are identified by examining: the components of the DDS design, the type of experiments run on the DDS, as well as the data collected from those experiments. As an example of this process, we constructed Tables 3 and 4 for the Circulating Semi-Infinite Reservoir Design, as used by Boskey.^{6, 13, 14, 18, 44–49} Similar tables could be constructed for the other systems presented, and we recommend going through such a process when designing and/or evaluating a DDS for studying biomineralization.

Finding a solution to the diffusion equation relies on knowing the initial conditions of the experiment and the boundary conditions under which the experiment is conducted. As an example, here is a solution (Eq. 5) that can be applied to the Circulating Semi-Infinite Reservoir Design, where the concentration of ions in the hydrogel sample is zero at the start of the experiment, the diffusivity of ions through the hydrogel is constant, the source (reservoir) of ions is semi-infinite and the hydrogel sample into which the ions are diffusing is also semi-infinite (Tables 3 and 4). Since it is a dynamic system, we start with Fick's 2nd Law (Eq. 3). For a complete description of how to solve such a problem, it is best to consult one of several textbooks available on diffusion.^{30–33, 43} Based upon the boundary conditions for the given problem, a solution based on an error-function is obtained (Eq. 5).

$$\begin{aligned} \frac{\partial c_i}{\partial t} = D_i \frac{\partial^2 c_i}{\partial x^2} \rightarrow c(x, t) = \frac{c_0}{\sqrt{\pi D_i t}} e^{-\frac{x^2}{4D_i t}} \rightarrow \int_0^\infty c(x, t) = c_0 \\ \text{if } u = \frac{x}{2\sqrt{D_i t}} \rightarrow c(x, t) = \frac{2c_0}{\sqrt{\pi}} \int_0^\infty e^{-u^2} du \\ c(x, t) = c_0 \operatorname{erfc} \frac{x}{2\sqrt{D_i t}} \end{aligned} \quad (\text{Eq. 5})$$

To make use of this equation, the boundary conditions, which provide the foundation for this solution, need to be reflected in the design of the DDS (Table 3). For example, the solution presented here only applies to diffusion in one-dimension. To fabricate a system that can be simplified to a one-dimensional problem, the gels must be confined in plastic tubes and their

connection to the system designed to restrict the diffusion for both ionic species to onedimension.

While the diffusivities, D , are assumed to be constant (Table 3), they are not truly independent variables. The movement of ions is strongly dependent on the interaction of those ions with their environment, the type of hydrogel,⁵ as well as any impurities in the hydrogel. Precise measurement of the diffusivity and the effect of these variables is difficult and for the purposes of this work need not be considered. As a result, the variables that contribute to the diffusivity eventually average out over the course of the experiment and can be (for the purpose of this work) assumed to be part of the “overall diffusivity” of the species in this system.

When determining or considering values of ion diffusivities, it is important to consider the counter-ion of the element that is diffusing and the temperature of the system. Different salts move at different rates according to the following relation:

$$D_{salt} = \frac{(z_+ + |z_-|)D_+ D_-}{z_+ D_+ + |z_-| D_-} \quad (\text{Eq. 6})$$

Where D_+ is the diffusivity of the cation and D_- is the diffusivity of the anion, z_+ is the charge of the cation and z_- is the charge of the anion. Therefore the diffusivity of a calcium “ion” is not necessarily universal to all calcium salts CaCl_2 , $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$, CaNO_3 , etc. Temperature also effects the speed of diffusion (+2–3% per °C from a value at 25 °C).⁸⁰ These differences in diffusivity can affect the reaction rate, supersaturation thresholds, and the stoichiometry of the resulting product. As such, counter-ions and temperature were not varied in the experiments described in Section 5.2.

Though it is clear that the original Circulating Semi-Infinite Reservoir DDS, as designed by Boskey, is a dynamic system and the fundamental design implies an semi-infinite reservoir system (Table 4), the experimental data presented a solution to the diffusion equation that is unique to a point source of infinite concentration (Table 4), diffusing in one dimension, from within an infinite sample.¹⁸ The discrepancy between the mathematically derived equation and the experimental results may arise from a poor circulation rate in the reservoirs, small reservoir volume, lack of reservoir replenishment or some additional unknown. Whatever the cause, Boskey’s demonstration of an experimentally driven solution to the diffusion equation is an example of how operational discrepancies with respect to the theoretical design can be compensated for using empirical data. This discrepancy, as well as others, is accommodated for and resolved by careful testing and re-evaluation of this particular design of a DDS. The boundary conditions for our reconstruction of this Circulating Semi-Infinite Reservoir Design and the strategies taken for that design are in Table 3.

5.2 Design and Evaluation of a Model System

As our model system we have chosen to optimize the design of a Circulating Semi- Infinite Reservoir dynamic DDS similar to the design shown in Fig. 5. Our initial interest in optimizing a DDS arose because we were interested in studying HA mineralization in the presence of surfaces and gradients of small-molecule mineralization inhibitors.¹⁶ Beginning with the DDS shown in Fig. 5, we discovered two characteristics that were challenging for our objectives: first, the timing of the mineral band formation was difficult to predict (varied amongst experiments and from tube to tube) and, second, the precise positioning of the precipitation events varied by as much as 1 mm from the center of the 6 cm tube in either direction. Neither of these challenges had posed a problem for the original applications, since they required precipitation to occur within a protein-laced, 0.2 cm thick band in the

center of the tube, during the course of the 5 day experiment. In addition, thick (0.3 cm – 0.67 cm wide) sections of gel, which were large enough to accommodate any slight positional variances in the mineral band, were analyzed for ion concentrations and mineral formation. In our desired applications, however, we needed to have mineral bands appear in more precisely defined locations and at more reproducible times from experiment to experiment.

To address these concerns, we made several key modifications to the original system (Fig. 5), including the addition of peristaltic pumps to circulate the fluids and stir plates to continuously mix the reservoirs (Fig. 7 and Figs. S1–S3). We also modified how the gelatin diffusion tubes were prepared and changed the angle of the circuit tubing connected to the reservoir to aid in the removal air bubbles from the system and maintain a constant gel/solution interface. Complete design details and a construction guide are provided in the online supporting information. We then designed three experiments to validate the chosen boundary conditions and fundamental assumptions we had with regard to our construction of the DDS (Tables 3 and 4).

5.2.1 Experiment I: Effect of the boundary conditions, $\Delta c_0=0$, $\Delta x_0=0$, and

$\Delta t_0=0$, on the kinetics of mineral precipitation—In order to apply Eq. 5, three fundamental boundary conditions must be maintained in all of the experiments. In a practical sense, the values of c_0 , x_0 , and t_0 can not change, or: $\Delta c_0=0$, $\Delta x_0=0$, and $\Delta t_0=0$. As such, the variables c_0 , x_0 , and t_0 (as defined in Fig. 1) must be tightly controlled using engineered safeguards to ensure reproducibility of the experiments.

The first boundary condition, $\Delta x_0=0$, demands that the dimensions of the gel sample do not change (through either swelling or shrinking) throughout the course of the experiment(s). The basic assumption is that the value of x_i and the values of x_0 and L at the boundaries are constant: $x_0(t)=0$, $x_L(t)=L$. However, if x_0 changes, the functions $x_0(t)$ and $x_i(t)$ would have to be 25 considered (these are cases where $x_0(t)=0$ and $x_i(t)=\text{constant}$).

The second boundary condition, $\Delta t_0=0$, demands that the starting time for diffusion must always be the same for every experiment. To consider all experiments normalized to the same start point, the reactant solution must make contact with the surface of each hydrogel in the experiment at the same time. In our system (Fig. 7), the start time t_0 of the experiment is controlled by using valves on the reservoirs, and a pump to introduce the reservoir fluid to each of the diffusion tubes at the same time or on a sufficiently short time scale. The “short” time scale required is found by applying the steady-state condition (Eq. 1) to the thickness of the gel edge at the interface layer. For this condition, we consider a gel edge to be 0.075 cm and a short time scale to be less than 15 minutes. In our system, new fluid from the reservoir is circulated by a peristaltic pump through the ~150 mL circuit in less than 3.5 minutes, making the lag time between the start of the pump and the connection of fluid to the ends of the hydrogel less than 5 minutes. New fluid from the circuit makes contact with the gel within 15 minutes. The movement of fluid through the system was monitored in an independent test using the circulation of different colored dyes. The 1 Hz oscillating flow of 42 mL/min is fast enough to equalize the system but slow enough to not dislodge the gel from the reaction tubes.

The third boundary condition, $\Delta c_0=0$, demands that there is no change in concentration of the reactants both at the solution/gel interface and in the source reservoir (the semi-infinite source condition). A constant value of c_0 at the solution/gel interface can be achieved by using either a single tube per large reservoir or by circulating the contents of the reservoir through a circuit of tubes at a rate faster than the concentration at the interface can be depleted by 0.1% of c_0 .³³ In our system with multiple tubes, the use of peristaltic pumps and

stir plates under each reservoir helps to maintain the value of c_0 at the solution/gel interface. The challenge of achieving a semi-infinite source is similar to the challenge of meeting the semi-infinite sample condition (see Section 4). A reservoir must be created such that, in either theory or practice, it can be considered semi-infinite. Two ways to achieve this condition are: use a reservoir sufficiently large in comparison to the sample specimen or change the solution in the reservoir frequently enough that there is negligible change in concentration over time.

In practice, the boundary conditions are not as straightforward as described above since values of certain boundary conditions are interdependent. For instance, the value of t_0 can change with the value of x_0 and c_0 . Since the value of t_0 is defined as the point in time at which the concentration c_0 crosses the point x_0 , if either c_0 or x_0 changes (or is different from tube to tube) then the value of t_0 will also change (assuming a constant diffusivity, D) (Fig. 6). If the solution/gel interface changes due to swelling or shrinking of the gel (see Section 3.4), x_0 will change, leading to an effective change in t_0 . Such structural changes of the hydrogel were not accounted for in the original design and use of the Circulating Semi-infinite Reservoir dynamic DDS. These structural changes may contribute to the observed discrepancies in the timing and the location of the precipitation bands, and may also contribute to deviations from the theoretical solution to the diffusion equation. For these reasons, we further examined the factors that lead to changes in x_0 and developed an approach to mitigate any structural changes that might be occurring in the gelatin tubes.

The thermodynamics that dictate changes in the hydrogel network at the solution/gel interface need to be correlated with the kinetics that dictate the movement of ions and water through the free volume of the hydrogel. To make this correlation, we use the same diffusion classifications (Cases, outlined below) used when considering structural changes in the diffusion of solutes and solvents in glassy polymers.³⁰ These cases clearly describe the various scenarios where the movement of a solvent/polymer interface is entangled with the diffusion of solutes through a polymer. In all cases, we clearly explain the similarities and differences between structural changes in glassy polymers and hydrogels.

Case I: The rate of diffusion is much slower than the rate of the structural changes: In the case for solute diffusion in glassy polymers this condition gives rise to a Fickian solution, since the polymer structure changes before the solutes even begin to diffuse, the diffusivity of the solute becomes the rate limiting step. In the case of a dispersive hydrogel network, however, the structural changes do not necessarily accommodate nor hinder diffusion, they in fact change the effective path length L of the gel. D_{eff} can still be considered constant, but the value of x_0 and/or t_0 will change depending on which variable is chosen to remain constant. One way to look at this case is to assume that changes in the structure will change the way x_0 or t_0 is determined, for instance t_0 will be a function of x_0 which may change before all the tubes on the system can be started within the required “short” time scale. Since in our use of the DDS, D is considered invariant, only x and t would be variable.

Case II: The rate of the structural change is very slow compared to the rate of diffusion: In solute diffusion in glassy polymers, this condition gives rise to non-Fickian diffusion, because the rate limiting step is the change in the polymer structure (the movement of the border between structurally changed and unchanged polymer). Simply put, the solutes need to wait for the polymer structure to change before they can move. For the case of a hydrogel, the network would neither swell nor shrink within the time frame of the experiment (making diffusion in a hydrogel Fickian), so this case would be an ideal situation for a DDS where $\Delta x_0 = 0$ and $\Delta t_0 = 0$.

Case III: The rate of diffusion is roughly equal to the rate of structural change: Again in this case, the diffusion through glassy polymers is non-Fickian. In this case both the diffusion rate and the rate of structural change are important and must be considered. In a hydrogel if t_0 is held constant, both D_{eff} and x_0 will vary with time, but if D_{eff} is considered constant then only x_0 will vary with time. This case is not a concern for t_0 since $\Delta t_0 = 0$.

For the evaluation of a DDS, since the swelling of the hydrogel network and the movement of ions are not monitored in real-time throughout the course of the experiment, it is difficult to determine which class of diffusion (either Case I or Case III) is taking place when the case is not explicitly Case II. We chose, therefore, to create a condition, which (for the most part) eliminates structural changes in the gel over the course of the experiment. To create such a situation and provide a Case II scenario there are a number of paths that could be taken (many have been explored but are not presented here). In the end, we adopted a “pre-hydration” strategy in which the gel/solution interface is pre-hydrated to the point where the gel ceases to shrink or swell anymore. For the gelatin tubes, the swelled ends of the hydrogel are then cut flush to the diffusion tubes before starting the experiment.

We then demonstrated the effects of controlling x_0 , t_0 , and c_0 from a phenomenological point of view, by observing the time and location of the precipitation event in the system (Experiment I). We tested four different experimental conditions, with varying boundary conditions: a non-circulating system with non-hydrated gels (Condition A), a non-circulating system with hydrated gel (Condition B), a circulating system with non-hydrated gels (Condition C) and a circulating system with hydrated gels (Condition D). The time at which the precipitation event could first be observed by eye in the gel tube was recorded for all 12 double-diffusion (DD) tubes (Fig. 8).

In examining the data from Experiment I, we can see how changing the conditions that affect the boundary conditions affect the timing and error in the timing of the mineral precipitation event in the gel (Fig. 8). In the optimized system (Condition D) where c_0 was maintained by circulating the reservoirs and the values of t_0 and x_0 were maintained by prehydrating (swelling) the gels, the precipitate appears after 71.42 hours, with no timing error variation amongst all 12 tubes. In contrast, in the un-optimized system (Condition A) where the reservoirs were not circulated and the gels were not pre-hydrated, precipitation is observed more than 24 hours after Condition D, with a large variation (up to 4 hours) among the 12 tubes.

This experiment highlights how critical the experimental conditions (and the resulting boundary conditions) are for determining the timing and reproducibility of the precipitation reaction. Circulation of the reservoir circuits (maintaining c_0) has the largest impact on when the precipitation occurs, whereas the pre-hydration of the gel (defining t_0 and x_0) strongly effects the reproducibility in the timing of the precipitation event.

5.2.2 Experiment II: Evaluating the semi-infinite condition for the sample length and reservoirs—A key boundary condition for the Circulating Semi-infinite Reservoir Dynamic DDS is the semi-infinite sample condition ($\lim_{(i \rightarrow L)} x = \infty$), and the semi-infinite reservoir condition, which is required for Eq. 5 to be applicable. The sample must be long enough in order to be considered semi-infinite for the time scale of the experiment.

In general, in order to quantitatively assess the validity of the assumed boundary conditions, an appropriate experiment to address each boundary condition must be performed. As an example, we designed an experiment using the semi-infinite sample length and semi-infinite reservoir size as our assessable boundary conditions. Since an evaluation of these two

conditions must be performed whenever working with a new or different hydrogel, we evaluated these conditions using 225 Bloom Type-A gelatin (10 w/v % in Tris buffer pH 7.4).

5.2.2.1 Experiment IIa: Evaluating the boundary condition of “semi-infinite sample length”:

For hydrogel-based DDSs the semi-infinite sample condition is a function of the diffusivity of an ion through a particular hydrogel, therefore each hydrogel (by type, concentration, strength, etc.) has a different semi-infinite sample condition. Using Eq. 4 and $D = 6.0 \times 10^{-6} \text{ cm}^2/\text{s}$ from the literature (the diffusivity of calcium in 10 w/v % 275 bloom gelatin in Tris buffer pH 7.4),¹⁸ it was found that a sample length greater than 7.4 cm would be required for a 5 day experiment to achieve the semi-infinite sample condition in this system. The semi-infinite condition can also be achieved using a sufficiently large enough sink tank (opposing reservoir) that would dilute the concentration of reactants to $<0.1\%$ of c_0 in a 12 tube system with a 6 cm sample length (3 mL tube), theoretically such a dilution can be achieved if the sink tank is 25 mL in volume (or continuously flushed away). The basis for this calculation can be found in the online supplemental material section S3.1.

In cases when a condition does not have an infinite end, as calculated by theory, it could be considered “quasi-infinite” if the error in measuring the concentration at the end, is such that there is no statistical difference between a result using a theoretically valid semi-infinite source versus a result using a theoretically invalid semi-infinite source. More simply put, if in theory it is not semi-infinite, but in practice there is no difference, then we will define it as quasi-infinite.

To confirm that the sample lengths are truly semi-infinite, an experiment was run using all Single-diffusion (SD) tubes, capped at one end, in the double-diffusion system and employing the “best practices” boundary conditions (Condition D) identified in Experiment I. We tested both 6 cm tubes¹⁸ (theoretically not semi-infinite, *i.e.* $< 7.4 \text{ cm}$) and 8 cm tubes (theoretically semi-infinite). After 5 days, the samples were removed from the system and the concentrations of ions along the length of the tube were determined and used to calculate diffusivities. In order to properly assess the validity of the semi-infinite boundary conditions, the measured concentration values were then compared with theoretically predicted values (using a calculated diffusivity and Eq. 5 for each condition).

The evaluation of the semi-infinite boundary conditions of the DSS hinges upon the measured concentration of ions in the hydrogel. More broadly, concentration analysis of ions in a hydrogel yields insight into: the threshold conditions, the map of ions across the specimen, the diffusivities of ionic species, and the validity of the boundary conditions. The concentration of ions in the hydrogel can be determined multiple ways.^{4, 10, 18, 19, 21} Here we use Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES), to determine the concentrations of both calcium and phosphate. The concentration data gathered from Experiment II was used to calculate the diffusivities of calcium and phosphate ions in 10 w/v% 225 Bloom Type-A gelatin gels (Table 5).

Diffusivities can be determined in a number of ways. The first method is one of brute force (for lack of a better term), where the concentration of an ion is determined in *multiple* slices along the path length of *multiple* samples of *varying* lengths, at *multiple* times.¹⁸ From *all* of this data, the applicable solution to the diffusion equation, which is unique to the boundary conditions of the experiment, such as the equation used for our system (Eq. 5), is used to solve for D at all points, given x and t :

1. Cut the gel into multiple slices (*e.g.*, 9) to obtain multiple points of x along the gel.

2. Measure the concentration of ions in each individual slice to obtain a concentration c for each point x (so 9 concentration values).
3. Repeat for multiple tubes (*e.g.*, 6 gels).
4. Repeat for multiple times t (*e.g.*, 10 time points).
5. Perform for each ion (2 ions).
6. Feed all 2160 data points into Eq. 5 and solve for D .¹⁸

There is another, easier method for calculating diffusivities that requires less time and without the need for high-end multivariable modeling programs (*e.g.*, Matlab[®] or Mathematica[®]). This method involves using the estimation of the diffusion penetration distance from a point source where the concentration has fallen to 25% of the concentration at x_0 ($x = 0$). This estimation, which is $x = 1.6 \sqrt{Dt}$, is unique to a diffusion equation where the error-function is the solution and the value of c is such that the solution of $\text{erf}(x)$ is $\frac{3}{4}$, or $\text{erfc}(x) = \frac{1}{4}$ and is unique to a semi-infinite point source.⁴³ In our solution to the diffusion equation, our concentration target is $c = c_0/4$. Similar approximations exist for other solutions to the diffusion equation.⁴³ This estimation simplifies the calculation of diffusion coefficients down to an algebraic fit. This straightforward method only requires one to:

1. Determine the concentration of three sections/slices (at the very least),
2. Plot those concentrations against the distance of each slice from the source,
3. Apply an exponential fit line and solve for x in such situations when for example $c = 25$ mM (in the case where $c_0 = 100$ mM).

The location of those three slices is dependent on the type and length of gel used, in cases where there is not an intuitive sense (or literature reference) of where the targeted c value might exist, it is a best practice to examine the concentration along the entire path-length of at least one gel to find three regions on or about where the targeted c value may lie. Using the three concentrations and three corresponding x values, a fit line is applied to find x where $c = c_0/4$. Once x is found for $c = c_0/4$ that value is plugged into $x = 1.6 \sqrt{Dt}$ at a constant time t . In general, for an experiment, the average from a set of 3–6 tube should be then taken and reported.

The concentration measurements taken in Experiment IIa show that both theoretically semi-infinite (8 cm) samples and theoretically finite (6 cm) samples yield similar (but not within statistical error) diffusivities for each calcium and phosphate species (Table 5). The calculated diffusivities were used to subsequently extrapolate the values of ion concentrations (using Eq. 5) at the end of their respective tubes and the center of the 6.67 mm wide end-slice (Table 5). These calculated values were then compared to measured values of ions within that end slice (Fig. 9) to confirm the validity of using the semi-infinite-based-estimation (erfc), for calculating the diffusivities). As a final comparison the target value of $c = 0.1\%$ of c_0 at $x = L$ (for each ion) serves as a benchmark; if the measured values lie under this benchmark then the sample clearly satisfies the semi-infinite sample condition.

Though the calculated diffusivities are similar for both the 6 cm and 8 cm calcium tubes, the data shows that the calculated value of the calcium concentration (the fastest ion for which the semi-infinite condition was originally calculated) in the 6 cm tube does not accurately predict the concentration of ions at the end of the 6 cm SD tubes (Table 5). The calculated diffusivities for calcium in the 6 cm tubes are therefore incorrect (not even considered “quasi-infinite”) because both the measured values and calculated values for a given location x are not consistent. In the 8 cm tubes for calcium, however, there is no statistical difference between the calculated/predicted calcium concentration and the measured calcium

concentration. Therefore the calculated diffusivities for calcium in the 8 cm tubes are correct, and the 8 cm tube can be considered semi-infinite for calcium. In contrast, when considering the ion concentration for phosphate, where a 6 cm tube is within the theoretical semi-infinite condition for that ion in 10 w/v % 225 Bloom Type-A gelatin, there is no statistical difference between the calculated ion concentration and the measured ion concentration for that condition (the data are self-consistent). Therefore the calculated diffusivities for phosphate in both the 6 cm and 8 cm tubes are correct and both a 6 cm tube and an 8 cm tube can be considered semi-infinite for phosphate.

A question not investigated was whether or not, statistically, it makes any difference having a SD tube capped or connected to a sink reservoir. Since the space on the bench top occupied by the 8 cm SD tubes was not an issue, we found that the semi-infinite condition could be satisfied by this extension in length, and there was no need to explore the use or efficacy of a sink reservoir. For some users, there may be situations (*i.e.*, certain kinds of diffusion tubes where capped ends are not an option) where they want to explore the option of using a sink reservoir.

In conclusion, 8 cm tubes represent a length that will ensure that the semi-infinite sample condition is maintained during single-diffusion experiments with either calcium or phosphate ions. Based on ion diffusivities in this hydrogel, we can then determine if a 6 cm tube used in the case of DD is semi-infinite up until the point of precipitation. After this point, any diffusion equation breaks down and the semi-infinite condition no longer matters. Using our calculated diffusivity for calcium from Table 5 ($D_{Ca} = 6.27 \times 10^{-6} \text{ cm}^2/\text{s}$), a 6 cm path length L for our DD tube, and Eq. 4, we solved for t and found that the moment when the semi-infinite condition breaks down is 75.37 hours. The data from Experiment I show that precipitation can happen as early as 71.42 hours. So even though an 8 cm tube is required for all SD tubes, a 6 cm tube is long enough to use for DD tubes, since the semi-infinite condition is still valid up until the time of precipitation.

5.2.2.2 Experiment IIb: Evaluating the boundary condition of “semi-infinite reservoir”:

To probe the semi-infinite source condition ($c > 99.9\%$ of c_0 for all time t) for the 1 liter reservoirs, sample concentrations of the reservoirs were taken at 0, 3, and 5 day time points, from the calcium and phosphate reservoirs in a DDS running only SD tubes (12 tubes per reservoir) (see Supporting information, Table S1). Analyzing only reservoirs from an all SD system assured no change in the effective concentration of the reservoir as a result of cross-contamination from the opposing reservoir while using DD tubes, over the course of the 5 day experiment. The theoretical calculations for the reservoir semi-infinite condition are based on the total flux of material during the time frame of the experiment, from the source reservoir into the gel, and from the gel into the sink reservoir, using values and conditions from the literature.¹⁸ Based on these calculations the source reservoir would need to be over 2 liters for the course of a 5 day experiment in order to be considered semi-infinite (for details of the calculation, see Supporting Information, Section S3), and the 1 liter reservoir ceases to be considered semi-infinite after 2.5 days (60 hours). If, however, there is no statistical difference between the measured concentration values and the calculated concentration values at a given time t , we can consider these source reservoirs as “quasi-infinite” sources.

Concentration analysis of the diluted (100:1) reservoir samples revealed two important results. First, the error of measurement for the ICP-AES instrument changes depending on the standards used for calibration, which are dependent on the target concentration one seeks to analyze. In our measurements of the reservoir samples we found that the error for a ~40 ppm calcium concentration is 0.1–0.4 ppm (0.25–1 mM calcium, undiluted), while for a ~36 ppm phosphorus concentration it is 0.06–0.25 ppm (0.2–0.8 mM phosphorus, undiluted).

Second, the concentration of reactants in the reservoirs did not effectively change from day zero to day three, while concentration analysis of the reservoirs on day 5 showed a ~2% drop in concentration in each reservoir (see Supporting Information, Table S1). The instrument did not have the resolution to determine at what point the value of the reservoir concentrations c drop below the semi-infinite reservoir condition. The measured reservoir concentrations after 3 days are the same as c_0 on day 0, even though the theoretical semi-infinite condition for a 1 liter reservoir ceases after 60 hours. In conclusion, the reservoirs can be considered quasi-infinite for the time frame of the experiment. Based upon this analysis, to avoid violating the quasi-infinite reservoir condition, for all experiments, we changed the 1 liter reservoirs just prior to the 3 day time point to maintain the effective c_0 concentration.

5.2.3 Experiment III: Empirically measuring the effect of changing x_0 , t_0 , and c_0 on the measured ion product—

The final experiment correlates changes in the measured concentrations of ions, that result from changes in x_0 , t_0 , and c_0 , to differences in the phenomenological precipitation events seen in Experiment I. This experiment not only examines the concentration of ions, but also the resulting ion product for calcium and phosphate (Fig. 10), for each system at a given time, to demonstrate the differences between two different experimental conditions. The ion product is directly related to the supersaturation, and thus amount of mineral created. The value and evolution of the ion product both temporally and spatially with the hydrogel is critical to the formation of crystals within the hydrogel.⁷⁰ The value of the ion product and the conditions required to reach supersaturation are dependent on the physical configurations and starting conditions of the experiment.⁹ As already discussed, controlling $\Delta x_0 = 0$, $\Delta t_0 = 0$, $\Delta c_0 = 0$ is essential for maintaining the boundary conditions of the system. Changes in these boundary conditions result in only small changes in the value of D for a given ion (data not shown), as well as slight differences in the measured concentrations for those ions (Fig. 11). Upon initial examination, these small changes and slight differences suggest that changes in the boundary conditions have little to no effect on the system as whole. These small differences, however, add up to significant changes in the value of the ion product in the system, at a given location, x , for a given time, t , (Fig. 1b), thereby affecting precipitation events. Phenomenologically, we saw this effect in Experiment I (Fig. 8) where changes in the boundary conditions lead to changes in the timing of the precipitation band.

Precipitation in a hydrogel is the foundation for studies that use a DDS, therefore the consideration of the threshold for such an event is important. Observing, controlling, and understanding the precipitation event is essential in order for work being done with a hydrogel-based DDS to be practical. The onset of precipitation signifies that a reaction has taken place. If the concentration values required to reach the supersaturation threshold can be accurately predicted, then the time and location of the precipitation event can also be accurately predicted. A possible pitfall when considering the use of the precipitation event in mathematically modeling the system, is that “Phenomenological-In” becomes “Phenomenological-Out” with no understanding of how and why. This experiment helps us to determine the how and why, by tying small variations in concentration gradients to the observed timing of the precipitation event (as seen in Section 5.2.1). The *in situ*, real-time monitoring of the spatial/temporal evolution of the ion product within the hydrogel is difficult but achievable using such systems such as dynamical interferometric analysis using a Mach–Zehnder configuration.⁹ Such configurations capitalize on changes in the refractive index and changes in the phase of incident light at points within the gel and extrapolates those changes into values of supersaturation at those points. In this work, we demonstrate the interrogation of concentrations, ion products and supersaturation, using post-experimental analysis.

In cases of static DDSs operating at sub-threshold conditions, a precipitation event signifies that the gel or whatever is in the gel is promoting mineralization.¹⁹ Ideally, control gels in such a static DDS will not mineralize over the course of the experiment, if they do mineralize then an error was made in either preparing the gels or the solutions.⁶¹ In this way, precipitation events can be used as a self-check of the experiment. In a dynamic DDS, the timing and shape of the precipitation event can yield insight into the effect that the gel or proteins in the gel are having on the formation of mineral, for instance, an early appearance of precipitate (relative to the control) can indicate a mineralization promoter, whereas a delay in precipitation can indicate an inhibitor.¹⁸

As precipitation occurs, a strictly diffusion-based model of any system by the diffusion equations breaks down due to crystal growth, local changes in the chemical potential, and local ion fluxes. As a result, the calculation and modeling of the system becomes increasingly difficult once precipitation occurs.^{18, 81–88} Based on our current observations and mathematical limitations in modeling the system, once precipitation has occurred, the study of the system becomes phenomenological.¹⁸

The empirical formula for HA is $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ and therefore the supersaturation for the formation of a precipitate in solution is dependent on a 9th order reaction ($[\text{Ca}]^5[\text{PO}_4]^3[\text{OH}]$). In a hydrogel, the supersaturation threshold is reduced to a two component reaction of $A + B = C$ where the supersaturation is the ion product $[A] \times [B]$, and the reaction is reduced to 2nd order, $AB \text{ mM}^2$, regardless of the true order of the reaction.⁸⁹ This reduction in reaction order is valid because precipitation under double-diffusion in a hydrogel obeys the equivalency rule rather than the ionic solubility law.⁸⁹ The empirical value of this supersaturation threshold is unique to the type of hydrogel used, the phase of the crystal being grown (ratio of ions, etc.), and the ions being used. A list of supersaturation threshold values for hydroxyapatite found in the literature (for a variety of hydrogels and conditions) has been compiled in Table 6.

For all of Experiment III, dynamic DDSs were run with varying boundary conditions with either exclusive DD tubes or a combination of DD and SD tubes. The two extremes from Experiment I, conditions A and D, were used. First, an all DD system was run under Condition D to establish the supersaturation threshold window for this gel (10 w/v % 225 Bloom Type-A gelatin) in this system (Table 6, Fig. 10). Once we established when precipitation occurred, this time point was used as a marker for the next experiment. In a comparison of the two conditions, two systems were run looking at each condition (Conditions A and D), 6 DD tubes were run, 3 for Condition A, 3 for Condition D, and 12 SD tubes were also run, 6 for calcium (Conditions A and D), 6 for phosphate (Conditions A and D). The two systems were stopped 2 hrs. $\pm$ 0.1 hrs. before precipitation was expected to occur for Condition D.

As a whole, the data from Experiment III show how the values of the ion product, used to measure supersaturation, change when x_0 , t_0 , and c_0 are changing for a given time, t , and a given location, x , due to changes in the experimental conditions. These experiments reveal the necessity of maintaining boundary conditions as a means to control the quantitative value of the ion product and the timing of precipitation in a DDS.

The first part of Experiment III established a supersaturation window for the optimized system (Condition D). An all DD system was run with 9 DD tubes (triplicates for each time point). Each set of three tubes was placed on the DD system at different time points (one hour apart from each other). When the first set of three showed signs of precipitation, all 9 DD tubes were taken off to determine the value of supersaturation at the time of precipitation and at time points up to two hours before precipitation was seen. Upon removal at 71.43

hours, only the first set of tubes showed signs of precipitation, the other two sets of tubes (+1 hour and +2 hour time points) were clear. After removal from the system, the tubes were placed in the refrigerator for 45 minutes to firm-up the gelatin before handling and cutting. After 45 minutes the two remaining sets of tubes began to show signs of precipitation but fainter than the first set of tubes (Fig. 10). The gels were cut and the center sections analyzed for ion content.

In the second part of Experiment III, both DD and SD tubes were run, a total of 12 tubes for each condition A & D, and the two systems were stopped at 69.36 hours (2 hrs. $\pm$ 0.1 hrs. before precipitation was expected to occur for Condition D). The measured concentration values for phosphate, for both Condition A and Condition D, are within error of each other for the SD tubes, and the measured values for calcium are similar to each other in the SD tubes (Fig. 11). The measured values of the ion product in all DD tubes is higher than would be expected based upon the recorded values of calcium and phosphate from the SD tubes (Fig. 11). This difference is most likely due to the formation of mineral product, not visible to the naked eye, in the diffusion tube. Comparing the calculated ion product for Condition A to Condition D (Fig. 12) reveals why the timing of band precipitation is so different between the two types of systems (Fig. 8). On average, the measured ion product of Condition A at 69.36 hours with no visible precipitation, was well below the minimum measured supersaturation-threshold value (19 mM^2) required for precipitation to be seen within the same timeframe in the optimized system (Condition D). Even with the wide variation in the statistical error of the measured ion product of Condition A, precipitation was not seen in any of these tubes. In contrast, the measured ion product for Condition D at 69.36 hours, just hours before visible precipitation, was well within the previously reported supersaturation-threshold window (Table 6), with the statistical error of the experiment lying above the lowest supersaturation value of that window.

In conclusion, the differences in the ion product for each condition clearly shows the importance of maintaining the boundary conditions of x_0 , c_0 , and t_0 in the performance of a hydrogel-based DDS. These differences in the value of the measured ion product translate to differences and variability in the timing of the precipitation events seen earlier in Experiment I (Fig. 8). In short, as the boundary conditions change/vary, so too do the conditions and timing of the mineral precipitation, which is strongly dependent on the concentration and ratio of ions at a given location in the hydrogel tube.

6. Conclusion

We have presented a survey of several established hydrogel-based DDSs that have been used to study the formation of calcium phosphate minerals as they relate to biomineralization. We have highlighted four systems, their application, the methods and rationale for their construction. In addition we have presented a protocol that can be used to either evaluate and/or construct and design a system for a specified application in studies of biomineralization. By exploring both the mathematical aspects regarding the governing diffusion equations, as well as the practical engineering and fabrication aspects, we hope to have made this class of diffusion systems more accessible. When designing a new DDS, one must keep in mind, the simpler the design, the less that can go wrong, but the less control you have over the system. The more complicated the DDS design becomes, the more control you have over the various mineralizing conditions, but the greater the chances for one small thing to make everything go wrong. For example, slight changes to the boundary conditions can lead to dramatic differences in the experimental outcomes, as demonstrated by Experiments I and III. In principle, for designing a DDS, the questions that must be asked and answered include: What are the intended boundary conditions? And how can they be maintained with high fidelity? Through this "Highlight" we hope we have provided the

reader with the necessary tools to ask and answer these questions, as well as to develop *de novo* DDSs.

In our explorations of the DDS, we have developed a detailed understanding of the operation of a DDS in the context of diffusion theory. By closely examining the underlying design criteria for all of these hydrogel-based DDSs, we have rediscovered what a powerful tool hydrogel-based double-diffusion can be for studying crystal growth and nucleation. We can now explore interesting questions in biomineralization such as:¹⁶ What is the role of substrates in mineralization within an ECM-like matrix? What are the effects regulating gradients of inhibitors on mineral formation within an ECM-like environment? And what are the effects of various types of hydrogels on both the crystal morphology and degree of mineralization in an environment where the rate of diffusion is the same? We hope that by sharing the insights we have found, new doors will be opened by those seeking to model mineralization within an extracellular matrix microenvironment, bringing even more exciting results to the field of biomineralization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge support from the Ithaca-Weill Seed Grant Program, the National Institutes of Health (DE018335 (LAE), DE04141 (ALB)), Cornell's Center for Materials Research (CCMR: a Materials Research Science and Engineering Center of the NSF, DMR 1120296), and the Cornell Nanobiotechnology Center (NBTC: a Science and Technology Center, ECS-9876771). In addition, LAE acknowledges support from the J.D. Watson Investigator Program (NYSTAR contract no. C050017). This work made use of shared facilities at CCMR with support from the National Science Foundation Materials Research Science and Engineering Centers (MRSEC) program (DMR 1120296) and through shared facilities at Cornell's Nanobiotechnology Center (NBTC) with support from the National Science Foundation STC Program (ECS-9876771). We thank Dr. Michael Rutzke (Department of Crop and Soil Science, Cornell University) for assistance with the ICP-AES data and Emily Asenath-Smith for bringing a fresh perspective to carefully reading and editing this "Highlight." Thank you to the pioneers of double-diffusion systems for the study of biomineralization, who through discussions over the years, have helped us appreciate the subtleties of the various designs. Finally, we thank all of our lab members who over the years have contributed to our understanding of the double-diffusion system including Hayat Taleb, Lyudmila Spevak, Dr. Dat Tran, and Dr. Ellen Keene.

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A) Static DDS (Fick's 1st Law)

B) Dynamic DDS (Fick's 2nd Law)

Fig. 1.

Representative illustrations of gels used in both static and dynamic double diffusion systems (DDS), the corresponding Fickian relations, and the characteristic plots of concentration, c vs. position, x . a) Static DDSs utilize Fick's 1st Law, where the flux, j , of ions is constant and c only varies with x . b) Dynamic DDSs utilize Fick's 2nd Law, where j changes with time and therefore c changes with both x and the time, t .

Fig. 2.

The Static Dual-Membrane Nested Tube DDS is used to study the effect various hydrogels have on the growth of calcium phosphate crystals. The nested tube design has a calcium reservoir nested within a phosphate reservoir, with the 15 μL hydrogel separated from the two reservoirs by a dialysis or ion-exchange membrane. Both reservoirs are set at a fixed volume and concentration (5–10 mM) at the beginning of the experiment, while the reaction occurs at 37°C. The concentrations of the reservoirs are chosen such that the supersaturation threshold condition is exceeded. The types of gels and proteins tested in this system have included: gelatin (bovine skin collagen, Type B 75 Bloom, 10%), bovine serum albumin (1%, 5%, 10%) poly(acrylamide) (10% with 0.5% crosslinker), agarose (0.5%), amelogenins (1%, 5%, 10%).^{11, 12, 20, 53–55, 57–59}

Fig. 3.

The Flowing Infinite Reservoir DDS is used to study the effects of extracellular matrix molecules on the nucleation of hydroxyapatite crystals. With an infinite reservoir design both the phosphate and calcium reservoirs are cycled using peristaltic pumps (1 per set of 3 assemblies) along the opposite boundaries of three separate, 1 cm long hydrogels. As the solution from the reservoirs flow past the hydrogel interface they empty into a waste collector at 1 mL/h per gel. The hydrogel is maintained in a steady state condition by the two reservoirs with a calcium concentration of 5.5–7.5 mM and a phosphate concentration of 5.5–7.5 mM. The interface between the hydrogel and the reservoirs is separated by dialysis membranes to keep target proteins within the hydrogel. The reaction takes place over the course of 5 days at pH 7.4 (Tris-buffer or HEPES buffer) at 37°C, and variety of hydrogels have been used (agarose 1% and 2%, type I native collagen 0.2%).^{19, 40–42}

Fig. 4.

A Thin Film Source DDS was used to examine the evolution of calcium pyrophosphate growth given various changes in mineralization conditions such as path length, pH (6–8), and starting concentrations. Experiments were run in test tubes 22 mm x 200 mm, with four layers of gel (275 Bloom gelatin 3–15% w/v), ~15 mL in volume (unless otherwise specified). One layer at either end serves as thin film sources for the ions of (calcium and pyrophosphate, 0.001–0.1 M) in the experiment with two ion-free layers of gel in the middle dictating the path length.^{10, 62–67}

Fig. 5.

The Circulating Semi-Infinite Reservoir DDS is used to study the effect of biomacromolecules sandwiched in a 10 w/v % gelatin hydrogel on the nucleation and growth of hydroxyapatite. 3 mL of a 10% w/v 275 Bloom Type A gelatin hydrogel in a pH 7.4 Tris buffer is cast into a 6 cm long pipette tube set between two 3–4 L reservoirs of constant volume. Each 3–4 L reservoir is circulated and degassed using either dry, CO₂-scrubbed air or compressed nitrogen, to maintain a constant concentration of 100 mM calcium and 100 mM phosphate. Each reservoir and ion circuit is of sufficient volume that they can be modeled as semi-infinite sources in the time frame of the 5 day experiment. The circuit tubing is constructed of vinyl tubing with polypropylene connectors. The connection

of the gel tubes to the circuit are made with thin walled vinyl tubing that is pre-stretched to provide a snug connection with the larger diameter gel filled diffusion tube.^{13, 14, 18, 44–49}

Fig. 6. The swelling and shrinking of the gel/solution interface has an effect on the boundary conditions of the system. With changes in x , the calculated effective values of t and D can also change.

Fig. 7. Optimized construction of the Circulating Semi-Infinite Reservoir Design. To create and maintain turbulent mixing, peristaltic pumps circulate each reservoir (42 mL/min) and stir plates (350 rpm) are placed under each reservoir. The reservoirs are constructed from 1 L media bottles modified with stopcocks attached at a 10° angle.

Experimental/Boundary Conditions

Fig. 8.

Timing in the onset of precipitation bands from the start of the experiment, in four different DDSs, Condition A: No reservoir circulation, no pre-hydration of the hydrogel; Condition B: No reservoir circulation, pre-hydration of the hydrogel; Condition C: Reservoir circulation, no pre-hydration of the hydrogel; Condition D: Reservoir circulation, pre-hydration of the hydrogel. All systems used 225 Bloom Type-A gelatin 10% w/v. The mode of the precipitation events for each condition is plotted with a horizontal line, with the error bars representing the maximum and minimum timing of precipitation in the tubes (12 each) under each condition.

Fig. 9.

Plot of the measured (■) and calculated (■) values of calcium and phosphate concentration in the end sections of two different lengths of diffusion tubes (6 cm and 8 cm) compared to the calculated value of c at $x = L$ (□) and the targeted theoretical value of 0.1% of c_0 shown as lines. (■ calcium, ■ phosphate). When the measured and calculated values are within error of each other, then the data are self-consistent and the assumed condition is considered valid (8 cm calcium and phosphate). When the measured value is equal to or less than the targeted value line, but the calculated value for c at $x = L$ is greater than the targeted value line, the condition is considered quasi-infinite (not seen here). When both the calculated value 47 for c at $x = L$ and the measured value at the end section are equal to or less than the targeted value line, then the condition can be considered semi-infinite (ex., calcium and phosphate 8 cm).

Fig. 10.

Scans of diffusion gels, cast from 10 w/v % 225 Bloom Type-A gelatin, from a Circulating Semi-infinite Reservoir DDS (run using Condition D). (a) Mineralized control gel with a dense mineral band formed after 5 days, (b) gels with the initial precipitation band seen at 71.43 hours, (c) gels removed ~1 hour before precipitation was seen and then left to sit for 45 minutes after being removed from the system, (d) gels removed ~2 hours before precipitation was seen (the faintest bands are circled for easy spotting) and then left to sit for 45 minutes after being removed from the system, (e) blank gel as cast and not placed on a DDS.

Fig. 11.

Measured calcium (■) and phosphate (□) values for the optimized (condition D) and un-optimized (condition A) systems in the circulating semi-infinite reservoir design after 69.36 hours (2 ± 0.1 hours before precipitation was expected). Values for calcium and phosphate from the SD tubes in both conditions were statistically similar. The main difference between the two conditions is the measured calcium concentrations for the DD tubes in each case. (†: The values for phosphate are within error of each other.)

[†]Electronic Supplementary Information (ESI) available: The ESI includes: a detailed construction guide for a Circulating Semi-Infinite Reservoir DDS; the experimental details for Experiments I–III; the Matlab code for calculating the semi-infinite reservoir condition, as well as supporting data for Experiment IIb. See DOI: 10.1039/b000000x/

Fig. 12.

Values of ion products (Ca x P) about location $x = 3$ cm. Shown are the average values of the ion product as calculated (Condition AC and DC) from concentrations in SD tubes and the values of the ion product as measured (Condition AM and DM) directly from DD tubes. The error bars represent the maximum and minimum possible (based on the standard deviation of the concentration values measured) values of the ion product. The average values for both the calculated and measured ion products for Condition D are within the supersaturation threshold window while only the maximum possible value for the ion product for Condition AM, falls within the supersaturation threshold.

Table 1

The key physical differences between static and dynamic DDSs. See Fig. 1 for further details.

Elements	Static Diffusion System	Dynamic Diffusion System
Path Length of Hydrogel (L)	L is sufficiently small that steady state is reached relatively quickly.	L is sufficiently large that steady state is not reached within the time frame of the experiment.
Reactant Concentrations (c)	c in the hydrogel is constant at a given point x for all time $t \gg L^2/D$.	c in the hydrogel is always changing for a given point x for all time t .
Flux (j)	The flux j of reactants through the hydrogel is constant.	The flux j of the reactants through the hydrogel is variable.
Concentration Gradients	Steady-state flux, j , provides a linear concentration gradient of reactants throughout the gel that does not change with time.	Concentration gradients of the reactants within the hydrogel are dynamic (j of reactants in, out and throughout the hydrogel is not constant) and changes with time.
Reservoirs	Since c at any point in the hydrogel is constant, the reservoirs act as <u>reserves</u> for the reactants in the hydrogel.	Since c at any point in the hydrogel is always changing, the reservoirs act as <u>sources</u> of reactants moving into the hydrogel.
Supersaturation Threshold	Operates at either sub- or superthreshold.	Operates throughout the threshold window.

Table 2

Criteria for choosing a DDS based upon the targeted application.

Desired Conditions	Static DDS	Dynamic DDS
Study mineralization within a hydrogel or ECM-like environment	*	*
Quantity of mineral is important.		*
Resolution of the reaction space is important (gradients of concentration and mineral).		*
Small number of tunable variables.	*	
Large number of tunable variables.		*
Questions That Need to be Answered		
Will it mineralize? (Yes/No) (Qualitative)	*	
How much will it mineralize? (Quantitative)		*

Table 3

Correlations between diffusion theory and real-world engineering considerations and components of double diffusion systems.

Conditions that Satisfy the Diffusion Equation:	
$c(x, t) = c_0 \operatorname{erfc} \frac{x}{2\sqrt{Dt}}$	Examples of Real-World Design Elements of the DDS
Concentration $c = c_0$ for location $x = 0$ and independent of time t , or all $t > 0$	Keeping the reservoirs at a constant semiinfinite volume (by changing/replenishing frequently), and constantly circulating, which keeps the surface concentration at the fluid/hydrogel interface constant.
Concentration $c = 0$ for location $x > 0$ at time $t = 0$	The hydrogels are made free of the diffusing ions and all gels are placed in contact with the ion solutions at the same time.
The sample path length L is semi-infinite	The hydrogel sample is made long enough such that it is considered semi-infinite throughout course of the experiment for all time, t .
The diffusivity D of the ions involved in the experiment are assumed to be constant (independent of: c, x, t)	The diffusivity of the ions are measured as a state function (after the experiment is over), it is therefore assumed that any and all contributing factors to the diffusivity are normalized by this singular measurement.

Table 4

Evaluation of the double diffusion system (DDS) used by Boskey¹⁸ where various aspects of the DDS are assigned to their respective meaning in diffusion theory.

Aspects of the DDS	What Those Aspects Mean
Used to examine the role of small amounts of non-collagenous proteins in the ECM.	A hydrogel environment is required.
Designed to measure both whether or not a mineral promotes or inhibits mineralization, and to what degree.	Dynamic DDS is required.
<ul style="list-style-type: none"> The sample length is 6 cm. The experiment lasts 5 days. The slowest ion (phosphate) diffusion rate is $3.9 \times 10^{-6} \text{ cm}^2/\text{s}$.¹⁸ The fastest ion (calcium) diffusion rate is $6.0 \times 10^{-6} \text{ cm}^2/\text{s}$.¹⁸ 	Using Eq. 1, Steady State Condition ($L^2/D_{min} = 107 \text{ Days}$) $\gg$ Length of the Experiment (5 Days) Therefore, the system is Dynamic .
<ul style="list-style-type: none"> Diffusion occurs in one dimension. Gels are made free of ions ($c = 0$ for $x > 0$ at $t = 0$). Reservoirs are kept at 3–4 L and circulated with N_2 ($c = c_0$ for $x = 0$ for all $t > 0$). Sample length of 6 cm is not semi-infinite ($6 \text{ cm} < \sim 4.6 (D_{max}t)^{1/2}$). D is assumed constant. 	<p>The theoretical solution to the diffusion equation is:⁴³</p> $c(x, t) = \frac{4c_0}{\pi} \sin\left(\pi \frac{x}{L}\right) e^{-\pi^2 D t / L^2} \text{ for } t \ll L^2 / (25 D)$ <p>The actual data from experiments as reported by Boskey et. al. reveals that the solution to the diffusion equation is:¹⁸</p> $c(x, t) = \frac{1}{2} c_0 \operatorname{erfc} \frac{x}{2\sqrt{Dt}}$

Table 5

Summary of data used to validate the semi-infinite sample boundary condition for a 10 w/v% 225 Bloom Gelatin Type A.

Reservoir Tube	Length	$c_0/4$ (μM)	Diffusivity ^b ($\times 10^{-6} \text{ cm}^2/\text{s}$)	Measured conc. at end section (μM)	Calculated conc. at end section using D (μM) ^b	Calculated concentration at $x=L$ (μM) ^b
Ca^{2+}	6 cm	25340	5.81 ± 0.17	407 ± 51	$1,160 \pm 118$	750 ± 85
Ca^{2+}	8 cm	25340	6.27 ± 0.21	93 ± 17	100 ± 19	60 ± 12
PO_4^{3-}	6 cm	29360	3.91 ± 0.41	251 ± 41	241 ± 130	129 ± 75
PO_4^{3-}	8 cm	29360	4.03 ± 0.48	55 ± 14^c	5 ± 2	2 ± 3

^aThe reservoirs contained either a calcium chloride solution ($c_0\text{Ca}^{2+} = 101.34 \text{ mM}$) or an ammonium phosphate monobasic solution ($c_0\text{PO}_4^{3-} = 117.45 \text{ mM}$).

^bThe diffusivities were calculated from the concentration of ions found in three sections of each diffusion tube (sets of 6), and then used to calculate the concentrations in the end sections of each tube, as well as the value of c at $x = L$.

^cThe error in measurement is on the order of the value measured, demonstrating the detection limit of the ICP-AES for this ion at this concentration.

Table 6

Literature values for the supersaturation threshold of HA as a function of hydrogel type and temperature.

Gel Type	Temperature	Ion Product [A]x[B]	Reference
Agar (0.5 w/v %) ^a	37°C	13.69 mM ² < c ² < 15.2 mM ²	21
Agar (1.0 w/v %) ^a	37°C	14.8 mM ² < c ² < 20.7 mM ²	21
Agar (5.0 w/v %) ^a	37°C	9.5 mM ² < c ² < 19.3 mM ²	21
Agarose (1 w/v %)	37°C	32 mM ² < c ² < 50 mM ²	5
Agarose (2 w/v %)	37°C	56.25 mM ²	5
Collagen (0.2 w/v %)	24°C	32 mM ² < c ² < 50 mM ²	5
Collagen (10 w/v %) ^a	37°C	13 mM ² < c ² < 14.4 mM ²	21
Gelatin (5.0 w/v %) ^a	25°C	16.8 mM ² < c ² < 24 mM ²	21
Gelatin (10 w/v %) ^a	25°C	15.2 mM ² < c ² < 25 mM ²	6
Gelatin (Type A; 275 Bloom; 5 w/v %)	24°C	32 mM ² < c ² < 50 mM ²	5
Gelatin (Type A; 275 Bloom; 10 w/v %)	20–25°C	^c 37 mM ² 36.85 mM ² < c ² < 41.5 mM ²	5, 18
Gelatin (Type A; 225 Bloom; 10 w/v %) ^b	20°C	^c 29.45 mM ² 18.92 mM ² < c ² < 37.82 mM ²	This work

^aPrecipitates in the gels were analyzed only after free ions were first removed from the gel.^bOriginal work presented in this Highlight (using average values from Experiment II and Experiment III).^cCalculated from determined diffusivities and recorded precipitation time.