

Spatiotemporal Formation of Calcium Phosphate Liesegang Patterns in Agarose-Gelatin Blend Hydrogels

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By
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Spatiotemporal Formation of Calcium Phosphate Liesegang Patterns in Agarose-Gelatin Blend Hydrogels

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We certify that we have read this thesis and that, in our opinion, it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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Liesegang patterns (LPs) are periodic structures formed by the concurrent diffusion and reaction of ions under non-equilibrium conditions, occurring both in natural systems and artificial settings. Since their discovery, several studies have been conducted to understand the mechanism and the effect of experimental parameters on pattern formation. Biomineralization, on the other hand, is a process that involves controlled nucleation, growth, and orientation of inorganic compounds by organic matrices. One of the common biomineralization processes in nature is the mineralization of calcium phosphates (CaP), which makes up an important proportion of human hard tissues, such as bones or enamel. The biological formation of calcium phosphates is hard to study since they are accompanied by many other complex biological processes. In this work, we aim to create an artificial system of CaP LPs in agarose-gelatin blends in varying proportions to mimic biological media. Such blends can serve as effective protein-polysaccharide scaffolds to simulate biomineralization environments due to their close resemblance to natural scaffolds. The chemical understanding of pattern formation, e.g., determining the identity and the morphology, can be useful in many applications, such as developing biocomposite ceramic materials, implants, and curing some diseases. Therefore, properties of CaP products formed in blend hydrogel samples were investigated under different experimental conditions, such as concentration, pH, and temperature, to understand how minerals form and adapt under varying parameters. The study reveals a hierarchical spatial arrangement of morphological within-band variety for the first time, alongside many other crucial observations based on the changes in experimental conditions. Thus, we believe that this work might offer new insights into understanding the evolutionary pathway of the biomineralization process, developing biomimetic composite ceramic materials, as well as contributing to the fundamentals of precipitation and mineralization processes.

ÖZET

Agaroz-Jelatin Karışımı Hidrojellerde Kalsiyum Fosfat

Liesegang Desenlerinin Uzam-Zamansal Oluşumu

Hatice Arı

Kimya, Yüksek Lisans

Tez Danışmanı: Bilge Baytekin

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Liesegang desenleri (LDler), doğada ve yapay ortamlarda, denge dışı koşullarda, iyonların eş zamanlı difüzyonu ve reaksiyonu sonucu oluşan periyodik yapılardır. Desen oluşumunun mekanizmasını ve deneysel parametrelerin desen oluşumu üzerinde etkisini anlamak için bu yapıların keşfinden günümüze kadar birçok çalışma yürütülmüştür. Diğer taraftan, biyomineralizasyon, inorganik maddelerin organik matrisler tarafından kontrollü çekirdeklenmesini, büyümesini ve oryantasyonunu içeren bir süreçtir. Doğada yaygın bulunan biyomineralizasyon süreçlerinden birisi de kemik ve mineraller gibi insanlara ait sert dokuların önemli bir bölümünü oluşturan kalsiyum fosfatların mineralizasyonudur. Kalsiyum fosfatların biyolojik oluşumu üzerine çalışmak, diğer birçok kompleks biyolojik süreç ile birlikte gerçekleştiğinden zordur. Bu çalışmada, biyolojik ortamları taklit etmek için agarozun ve jelatinin değişen oranlarda karışımlarında, yapay kalsiyum fosfat Liesegang desenlerinin oluşturulması amaçlandı. Bu tür karışımlar, doğal iskeletlere olan benzerliklerinden dolayı biyomineralizasyon ortamlarını taklit etmek için etkili bir protein-polisakkarit iskeleti olarak görev yapabilir. Desen oluşumunun kimyasal anlayışı, kimliği ve morfolojiyi belirlemek gibi, biyokompozit seramik materyallerin ve implantların geliştirilmesi ve hastalıkların tedavisi gibi birçok uygulamada faydalı olabilir. Bu yüzden, minerallerin değişken parametrelere göre nasıl oluştuğunu ve adapte olduğunu anlamak için konsantrasyon, pH, ve sıcaklık gibi farklı deneysel koşullar altında ve hidrojel karışımı örneklerde oluşan kalsiyum fosfat ürünlerinin özellikleri incelendi. Araştırma, bant içi morfolojik değişimlerin olduğu hiyerarşik uzamsal düzeni ilk kez göstermesinin yanı sıra deneysel koşullara bağlı diğer birçok önemli gözlemi de ortaya çıkarmıştır. Bu yüzden, bu çalışmanın biyomineralizasyon sürecinin evrimsel yolunu anlamak, biyomimetik kompozit seramik materyaller geliştirmek, ayrıca çökeltme ve mineralizasyon süreçlerinin temellerine katkıda bulunmak için yeni görüşler sunabileceği düşünülmektedir.

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TABLE OF CONTENTS

1. Introduction	1
1.1 Biomineralization	1
1.1.1 Calcium Phosphates: Crystal Structures and Their Identification	1
1.1.2 Calcium Phosphates: Crystal Morphology	4
1.1.3 Calcium Phosphates: The Effect of Experimental Conditions on the Product Formation	6
1.2 Liesegang Patterns (LPs)	8
1.2.1 External Factors Affecting the Pattern Formation	10
1.2.2 Biomimetic Control of Pattern Formation	12
1.3 The Role of Gel Medium in LPs and Biomineralization	12
1.4 Calcium Phosphate Liesegang Patterns	14
1.5 The Purpose of the Work	17
2. Materials and Methods	19
2.1. Materials	19
2.2. Choosing the Gel Blends as the Media for CaP formation.....	19
2.3 Gel Preparation	20
2.3.1 Preparation of the Agarose-Gelatin Blend Blank Samples	20
2.3.2 Preparation of the Gelatin and Agarose (control) Samples and the Agarose-Gelatin Blend Samples for CaP LP formation.....	20
2.3.3 Preparation of the Gelatin-Collagen and Agarose-Collagen Blend Samples for CaP LP formation	23
2.3.4 Magnesium Hydroxide Control and Blend Samples	24
2.4 Experiments for CaP LP formation at varying Temperatures	24
2.5 Experiments for CaP LP formation under varying pH conditions	25
2.6 Solution Synthesis of CaP	27
2.7 Ion – Gel Interaction Experiments.....	27
2.8 Characterization and Analysis	28

2.8.1 Characterization	28
2.8.2 Image Analysis.....	29
3. Results and Discussion.....	31
3.1. Magnesium Hydroxide and Calcium Phosphate LP Formation in Blend Hydrogels....	31
3.2. The Effect of Synthesis Method on the Phase and Morphology of the CaP Product....	36
3.2.1 CaP Formed via Solution Synthesis.....	36
3.2.2 CaP LPs Formed in Agarose, Gelatin, and Agarose-Gelatin Blends via Reaction-Diffusion	38
3.3 Experiments for CaP Formation under Different Experimental Conditions	47
3.3.1 The Effect of Gel and Outer Electrolyte Concentration on Crystal Morphology	47
3.3.2 Experiments for CaP LP Formation at Varying Temperatures.....	48
3.3.3 Experiments for CaP LP Formation under Varying pH Conditions	51
3.3.4 The Effect of Functional Groups on Morphology	60
3.4 CaP LPs in Agarose-Collagen and Gelatin-Collagen Blends.....	63
3.4.1 Pattern Formation in Gelatin-Collagen Blend Systems	63
3.4.2 Pattern Formation in Agarose-Collagen Blend Systems.....	64
3.5 Potential Applications of CaP LPs in Blend Samples	65
4. Conclusion.....	67
References	70

LIST OF FIGURES

Figure 1. The most studied phases of calcium phosphates, their chemical properties, and common areas of use. The color code represents the relative solubilities of each phase. The Ca/P ratios and the pK_{sp} values are given under the phases' letter abbreviations. The arrows show which phases can transform into one another when proper conditions are provided. 2

Figure 2. a) Changes in ΔG^* , Gibbs free energy for epitaxial growth of HAp as a function of supersaturation (σ), in which ΔG_{mis}^* , $(\sigma_{surf})_c$, and m are the activation energy barrier for mismatched secondary crystal, critical surface supersaturation required to form a secondary mismatched nucleation, and mismatch angle between parent and daughter crystals, respectively. Reproduced from Ref [38]. b) The scheme of the most common crystal morphologies of the different CaP phases shown in **Figure 1**. 5

Figure 3. a) Solubility isotherms of several CaP phases as a function of pH and the logarithm of calcium and phosphate concentrations. b) Solubility of different phases depending on pH. The graph also exhibits equilibrium between phosphate ions at different pH values. Figures reproduced from Ref [13]. 6

Figure 4. Magnesium hydroxide patterns produced in a) 1D, and b) 2D setups. c) CaP patterns produced in 3D LP setup, reproduced from Ref [62]. d) Empirical rules followed by LPs: time law, width law, spacing law, and Matalon-Packter law. x_n and x_{n+1} represent the position of consecutive bands, whereas w_n and t_n denote the width of the band and time, respectively. A_0 and B_0 are the initial concentrations of the outer and inner electrolytes. $1+p$ is the spacing coefficient, and β_0 and γ_0 are constants. 9

Figure 5. a) CaP LPs in agarose medium ($Na_2HPO_4 = 0.015$ M, $CaCl_2 \cdot 2H_2O = 1.5$ M) at different temperatures. b) The decrease in the spacing coefficient with increasing LP formation temperature. 11

Figure 6. Possible chemical interactions between calcium ions, phosphate, and functional groups of polyamides that can act as nucleation centers, reproduced from Ref [85]. 14

Figure 7. The illustration of the experimental setup for the preparation of the gel blends and LP patterns. The gels are dissolved separately, while the inner electrolyte ($CaCl_2 \cdot 2H_2O$, 0.020 M) was added to the gelatin solution. Gelatin/inner electrolyte solution was then added dropwise to agarose solution. After mixing, the final solution was transferred to a glass Pasteur pipette. The solution was allowed to gelate, and after the gel formed, the outer electrolyte (Na_2HPO_4 (aq), 0.50 M) was added into the pipette, to the top of the gel to start the LP formation. 22

Figure 8. The samples before and after outer electrolyte addition (left). CaP LPs formed in gelatin, agarose, and an agarose/gelatin blend. (Polymers and the polymer blend = 5.0% (w/v))	23
Figure 9. The setup of the home-made temperature control device consisting of a Peltier element and an Arduino Uno.	25
Figure 10. The effect of the drying method on the samples' morphology. The vacuum drying method preserves distinct regions of samples, whereas the freeze-drying method homogenizes gel medium and makes it impossible to differentiate separate regions. Therefore, the former was used in the SEM sample preparation.	28
Figure 11. a) The graph of the gray value profile of bands vs. the distance of bands in pixels from the gel-solution interface. b) The same graph created in a home-made MATLAB code with the “find peaks” function. c) The graph of the natural logarithm of band position and band number. The slope gives the spacing coefficient.	29
Figure 12. Magnesium hydroxide LPs prepared in gelatin, agarose, and blend hydrogel samples. Spacings and widths of bands changed based on gel composition. G= % (w/v) gelatin and A=% (w/v) agarose.	31
Figure 13. SEM micrographs of magnesium hydroxide LPs in different hydrogels/hydrogel blends shown in Figure 12. The ratios of gelatin/agarose are in a) 5.0% to 0, b) 1.0% to 4.0%, c) 2.0% to 3.0%, d) 2.5% to 2.5%, e) 3.0% to 2.0%, f) 4.0% to 1.0%, g) 0 to 5.0%. The second forming band in each sample was imaged for comparison. The samples displayed similar, platelet-like morphologies. The direction of the arrows indicates a decreasing concentration of gelatin. Only in the sample prepared through the wet-chemical precipitation method, shown in (h), is the morphology slightly more irregular than the others. The scale bar is 10 μm for a) to g) and 2 μm for h).	32
Figure 14. a) CaP LPs prepared in gelatin, agarose, and blend hydrogel samples. Spatial formation and width of bands changed based on gel composition. G= % (w/v) gelatin and A=% (w/v) agarose. b) A single CaP LP in a blend hydrogel. The image shows different types of bands formed in the same sample and different regions of a split-fin band. c) The graph exhibiting the changes in the spacing coefficient based on gel composition.	33
Figure 15. LPs forming in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium with different ion concentrations, in which $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was the inner electrolyte, and Na_2HPO_4 was the outer electrolyte in varying concentrations. The concentrations at the top of each box belong to the inner electrolyte concentration, and the ones given under each sample are the concentration of the outer electrolyte solution.	35

Figure 16. CaP samples prepared through the wet-chemical precipitation method under a-d) high Na_2HPO_4 , and e-h) low Na_2HPO_4 concentration conditions. $[\text{CaCl}_2 \cdot 2\text{H}_2\text{O}] = 0.020 \text{ M}$, $[\text{Na}_2\text{HPO}_4]$ is given below the micrographs (in red). Ca/P ratios determined by EDX are given below the images. f) and g) are zoomed areas highlighted with a red circle in e). i) and j) show the IR spectra of the samples.....	37
Figure 17. The micrographs of CaP formed in LPs in agarose or gelatin. a) and b) belong to the spire and plateau regions of 5.0% (w/v) gelatin samples, respectively. c) and d) are the micrographs of the last band above continuous precipitation in 5.0% (w/v) agarose sample. e) is the product in the continuous precipitation region observed in the middle part of 5.0% (w/v) agarose sample. Inset: the inside of spherical structures. f) is the first band forming after continuous precipitation. The scale bar is 10 μm for b), c), d), e), and f), 5 μm for a), and 4 μm for the inset figure of e).....	39
Figure 18. The IR spectra of a-c) 3.0% (w/v) agarose + 2.0% (w/v) gelatin blank gel samples, d-f) 3.0% (w/v) agarose + 2.0% (w/v) gelatin samples with 0.020 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ across different parts in the gel (named as bottom, middle, and top).	40
Figure 19. The micrographs of crystals forming in different regions of an LP band and varying gel concentrations show the various morphologies of CaP obtained within a single band in the LP. G and A represent % (w/v) gelatin and % (w/v) agarose, respectively. The red framing shows different regions of 3.0% (w/v) agarose + 2.0% (w/v) sample, while the yellow framing demonstrates how the particle size and number change as a function of blend composition. The scale bar is 5 μm for spire regions and 10 μm for plateau, void, and spike regions.	42
Figure 20. Spatiotemporal formation of LPs in 4.0% (w/v) agarose + 1.0% (w/v) gelatin blend sample. a) and b) exhibit crystal morphologies of distinct regions in different generations of bands. The scale bar is 5 μm for the spire and 10 μm for the other regions.	43
Figure 21. The IR spectra of a) 3.0% (w/v) agarose + 2.0% (w/v) gelatin and b) 4.0% (w/v) agarose + 1.0% (w/v) gelatin. The new peaks/shoulders (circled on the spectra) arise from the CaP product.	45
Figure 22. XRD diffractogram of samples formed in 3.0% (w/v) agarose + 2.0% (w/v) gelatin. Comparison of a) the sample diffractogram with possible phases present in the sample and b) distinct regions of different bands. 1 refers to the last forming band, while 2 corresponds to the second-to-last band. Reference patterns are OCP: 00-074-1083 (ICDD), HAp: 00-009-0432 (ICDD), CDHAp: 01-073-2657 (ICSD), DCPD: 01-072-1240 (ICSD), and TCP: 00-009-0348 (ICDD).	46

Figure 23. The morphology diagram displaying how gel and outer electrolyte concentrations affect the particle morphology in different regions of a band. The morphologies of crystals are color-coded. Yellow, green, red, and blue represent agglomerated-crumpled morphology, flake-like and star-like morphology, nanoflower structures, and flake-like morphology in lower density with star-like morphology, respectively.	47
Figure 24. CaP LPs prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium at different temperatures.	49
Figure 25. SEM micrographs of crystals formed in different regions of a band (in 3.0% (w/v) agarose + 2.0% (w/v) gelatin) at 4.0 °C, 25 °C, and 37 °C. The purple and red frames are used to show uniformity of particle shapes and an increase in the number and size of particles, respectively. The scale bar is 5 µm for the spire and 10 µm for the other regions.	50
Figure 26. Possible interactions between the ions with the gel structure and the CaP particle formation. The arrows indicate the direction of diffusion.	52
Figure 27. The chemical structures of gelatin (top) and agarose (bottom).	52
Figure 28. CaP LP samples produced under different outer electrolyte and gel pH conditions. pH of the gel is a) 4.0, b) 5.5, and c) 12. pH of outer electrolyte solutions is 5.5, 7.0, 9.0, and 12.	54
Figure 29. Particle morphologies of CaP samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin gel with a pH of 4.0 and varying outer electrolyte concentrations. (Please refer to Section 2.4 for abbreviations.) Numbers 1, 2, and 3 symbolize different parts of the same band, and ‘ppt’ denotes random precipitates formed after the LP bands. The scale bar is 5 µm for OE7/G4-2, OE7/G4-ppt1&2, OE9/G4-ppt1, and OE10.5/G4-ppt1, and 10 µm for the remaining micrographs.	55
Figure 30. SEM micrographs of the product formed in CaP LPs prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium with pH 5.5. ppt stands for random precipitate. “*” means the last split-fin band of the OE7/G5.5 sample. (Please refer to 2.4 for abbreviations.) The scale bar is 5 µm for spire and ppt regions and 10 µm for the remaining images.	57
Figure 31. SEM micrographs of the product formed in CaP LPs prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium with pH 12. 1 and 2 represent different regions of continuous precipitate, and 3 corresponds to the sharp front forming after the continuous part (Please refer to 2.4 for abbreviations.) The scale bar is 10 µm.	58
Figure 32. Photos and SEM micrographs of the product in CaP LP samples prepared in a) 1.0% (w/v) agarose, and b) 1.0% (w/v) agarose + 0.018% (w/v) gelatin media. Red-framed and blue-	

framed regions display the LP and continuous precipitation regions, respectively. The scale bar is 10 μm 61

Figure 33. The IR spectra of a) gelatin samples either doped with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ or prepared with diffusion of Na_2HPO_4 in gel medium, b) agarose samples doped with different concentrations of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and c) agarose samples prepared with diffusion of Na_2HPO_4 in gel medium. A and G correspond to % (w/v) agarose and % (w/v) gelatin, respectively. Any changes or regions expected to have changes are marked with either arrows or different symbols. 62

Figure 34. CaP LPs generated in agarose and gelatin control samples, and gelatin-collagen and agarose-collagen blends. G, A, C1, and C13 stand for % (w/v) gelatin, % (w/v) agarose, % (w/v) type I collagen, and % (w/v) type I&III collagen, respectively. “*” symbol represents that calcium is doped to collagen instead of gelatin. 63

Figure 35. SEM micrographs of the product formed in CaP LPs prepared in gelatin-collagen blends. G, C1, and C13 stand for % (w/v) gelatin, % (w/v) type I collagen, and % (w/v) type I&III collagen, respectively. “*” symbol represents that calcium is doped to collagen instead of gelatin. The scale bar is 5 μm for spire regions and 10 μm for plateau regions. 64

Figure 36. SEM micrographs of CaP LPs produced in agarose-collagen blends. A, C1, and C13 stand for % (w/v) agarose, % (w/v) type I collagen, and % (w/v) type I&III collagen, respectively. “ppt” stands for continuous precipitation region in the middle of the samples. The scale bar is 10 μm 65

LIST OF TABLES

Table 1. Literature summary of CaP biominerals produced in various gel media. The table shows varying experimental conditions and synthesis methods that lead to the formation of different crystal morphologies.	16
Table 2. The amounts of polymers used to prepare corresponding blend concentrations.....	21
Table 3. Formation of LPs under different outer electrolyte and gel pH conditions. The “-” sign means that the reaction did not produce a visible precipitate, while the “+” sign refers to systems producing LPs. When the gel pH was set to 4.0, patterns formed relatively weakly compared to other systems. ppt and cts are short-hand notations for ‘random precipitate formation’ and ‘continuous band formation’, respectively.....	26
Table 4. Ca/P ratio of different regions of control and blend samples.....	44
Table 5. Ca/P ratio of different regions of samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium at different temperatures.....	51
Table 6. Ca/P ratio of different bands of samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium under different gel and outer electrolyte pH conditions. 1, 2, and 3 symbolize different bands (or regions in the case of gel pH 12), while ppt stands for random precipitates formed after bands. (Please refer to 2.4 for abbreviations.).....	59
Table 7. Ca/P ratio of different bands of samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium under different outer electrolyte pH conditions. ‘ppt’ stands for random precipitates formed. (Please refer to 2.4 for abbreviations.)	59

LIST OF ABBREVIATIONS

LP	Liesegang pattern
CaP	Calcium phosphate(s)
DCPD	Dicalcium phosphate dihydrate
DCPA	Dicalcium phosphate anhydrous
ACP	Amorphous calcium phosphate
TCP	Tricalcium phosphate
TTCP	Tetracalcium phosphate
OCP	Octacalcium phosphate
HA/HAp	Hydroxyapatite
CDHA/CDHAp	Calcium-deficient hydroxyapatite

CHAPTER 1

1. Introduction

1.1 Biomineralization

Biomineralization refers to a natural or artificial process [1] in which organic matrices substantially contribute to the nucleation and growth mechanisms of inorganic minerals [2], as well as the orientation of forming crystals [3]. Several minerals produced through biomineralization can be utilized in the design of biomimetic materials [4], medical imaging [5], targeted drug delivery systems [6], biosensors [7], and photonic devices [8]. To exemplify, diatom biosilica has been used in several areas, such as wearable devices [9]. Similarly, several species using calcium, magnesium, or iron as cations, and hydroxide, carbonate, and phosphates as anions are also useful biominerals for different functionalities [10]. Nonetheless, calcium phosphates (CaP) are among the most studied biominerals due to the abundance of phases [11] and their hierarchically ordered presence in the bones of vertebrates [12]. They are of great use, especially in drug delivery and bone regenerative studies, due to their desirable intrinsic properties such as osteoconductivity and bioactivity [13]. The chemistry aspect of biomineralization studies, especially those focusing on calcium carbonate (calcite) and phosphates, can be classified under three main categories: 1) characterization and composition, 2) in vitro modeling of biomineral formation to reveal the role of biomacromolecules in nucleation and crystal growth processes, and 3) the methodological advances to achieve desirable or controllable morphology and polymorph formation. All these categories contribute to next-generation biocomposite material design [14]. Understanding the key chemical aspects of biomineralization is crucial for comprehending how it occurs in natural systems. Thus, formed phases should be identified, and the effect of ambient conditions on biomineral formation should be determined to provide a better understanding of the biomineralization process.

1.1.1 Calcium Phosphates: Crystal Structures and Their Identification

Calcium phosphates can form several phases when specific ambient conditions are met. Different phases of CaP are utilized in several applications, especially in food [15], pharmaceutical [16], and biomedical industries [15][16]. Among these applications, using CaP structures for biocomposite material design stands out. When prepared in hydrogel media, CaP might contribute to the extracellular pools, similar to the case of the activity of bone biominerals

[17]. The extracellular matrix of bone has been reported to consist of collagen, non-collagenous proteins, and hydroxyapatite [18]. Thus, a hydrogel medium that resembles these proteins or polysaccharide components, e.g., agar [19], gelatin [20], or collagen [21], has been employed to obtain synthetic biomineralization of CaP. The media in which the biomineralization takes place has a direct effect on the identity and crystal structure of the formed CaP. Therefore, it is necessary to identify these different forms and structures (**Figure 1**).

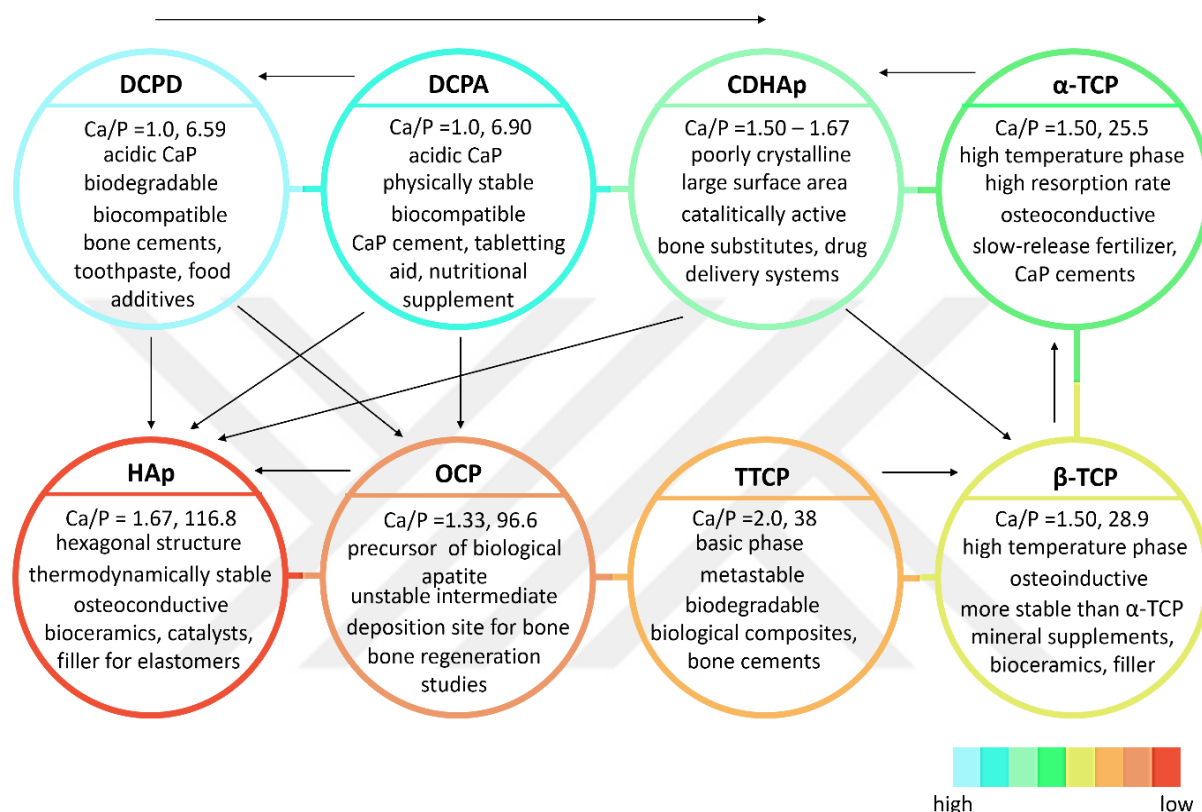


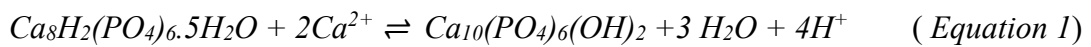
Figure 1. The most studied phases of calcium phosphates, their chemical properties, and common areas of use. The color code represents the relative solubilities of each phase. The Ca/P ratios and the pK_{sp} values are given under the phases' letter abbreviations. The arrows show which phases can transform into one another when proper conditions are provided.

Amorphous calcium phosphate (ACP, $\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$) is a non-crystalline form of calcium phosphates forming under supersaturation conditions [13], which can transform into other crystal structures under specific pH conditions [13]. The acidic medium promotes the formation of DCPD, whereas HAp formation can be induced in basic reaction conditions [22]. ACP tends to form in earlier stages of precipitate formation due to lower surface energy than other phases [23]. It was hypothesized that randomly packed spherical Posner's clusters ($\text{Ca}_9(\text{PO}_4)_6$) with around 0.9 nm size are the building blocks of the ACP structure [24]. In later stages, these clusters induce the formation of HAp stepwise. The precipitation of ACP has been proven to be highly dependent on experimental factors, including pH, temperature, and

precursor concentration [13][23]. Although different Ca/P ratios are attributed to ACP, the sources mostly come to an agreement that the Ca/P ratio of the ACP structure varies between 1.20 to 2.20. This might be explained by the fact that ACP contains variations in chemical composition resulting from pH differences [23].

Hydroxyapatite (HAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), among all the other phases, is known to be the most thermodynamically stable phase [25] with very low solubility [13] and constitutes approximately 70% of human bone [23]. Apart from its biological importance, HAp is commonly used as a green catalyst in a wide range of catalytic processes because it has high thermal and mechanical strength [26], high porosity [23][26], chemical stability [26], and selectivity [26]. HAp has other applications, too [26][27]. Its high crystallinity [13] with a similar Ca/P ratio (1.67) [23] to the enamel (1.63) or bone (1.71) [13] makes it a suitable substitute material for human hard tissue [27]. Many of the other phases of CaP (**Figure 1**) can transform into HAp under proper conditions, as it is the most thermodynamically stable phase.

Octacalcium phosphate (OCP, $\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$) is a metastable phase of calcium phosphate biominerals and one of the primary precursors of biologically formed HAp [13]. *Equation 1* demonstrates how metastable OCP hydrolyzes to produce HAp [28]. Among other calcium phosphate phases, OCP is hypothesized to have a higher affinity towards organic molecules [29]; in other words, it can interact more readily or strongly than other structures due to its crystallographic planes and relatively empty hydrated layers [29]. The solubility of OCP is comparatively higher than that of HAp, which makes it a better candidate for drug release systems [29]. Some studies report that OCP repairs defects in bone structure [30] and enhances bone formation [22][29][30].



Dicalcium phosphate dihydrate (DCPD, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) and dicalcium phosphate anhydrous (DCPA, CaHPO_4) are the other well-known phases of calcium phosphates [23] formed in acidic media. Both structures are more soluble than other phases [13] and can be classified as precursors of the more stable phases, such as HAp [31]. Successful crystallization of DCPD is reported between pH 2.0 and 6.5 [13]. The structure of DCPD contains two water layers; one is a highly ordered layer that is a part of the crystal structure, and the other shows layering in the perpendicular direction [13]. DCPD can transform into DCPA at slightly elevated temperatures (above 80 °C) [13]. DCPD formation is common in pathological

calcifications, such as dental calculi or urinary stones [13]. DCPA and DCPD structures are extensively used in industrial applications such as cements [23][31].

Although it is comparatively difficult to identify the crystal structures of CaP because of their structural similarity, several characterization techniques are available for phase identification. Undoubtedly, X-ray diffraction (XRD) analysis is the most accurate and reliable method, as it defines characteristic crystal lattices [32] and the crystallinity of each phase [33]. XRD analysis can determine atomic unit cell parameters [33], providing a fingerprint diffractogram for separating phases with a quantitative analysis.

Energy-dispersive X-ray (EDX) analysis can be a beneficial tool for assessing the crystal structure by offering information on the Ca/P ratio of the sample [34]. Fourier-transform infrared (FTIR) spectroscopy can also provide evidence for crystal structures based on the vibrations of phosphate groups present in the structure of a specific phase or hydration level of each structure [32][33]. As distinct CaP phases contain groups such as hydrogen phosphate (HPO_4^{2-}) or hydroxide (OH^-), IR spectroscopy might be utilized to identify those phases having these groups [35]. Thermogravimetric analysis, or TGA, is a relatively less commonly used method for crystal structure identification than EDX and IR spectroscopy analysis. Nevertheless, it is an invaluable complementary technique for identification, especially for structural water phases [33], like in DCPD, OCP, and ACP. It can also help identify the crystal structures based on their decomposition temperature [35].

1.1.2 Calcium Phosphates: Crystal Morphology

Crystal morphology refers to the shape of particles determined by the internal structure of the compound and the relative growth rate of planes binding to the crystals [36]. Several methods, including hydrothermal and wet-chemical precipitation methods [37], have been extensively used to prepare different CaP crystals and their various morphologies.

Varied shapes and sizes of CaP particles can be obtained by arranging the properties of the reaction medium. For instance, it has been repeatedly reported that ACP forms spherical particle agglomerates under supersaturation conditions [38], where nuclei form and further growth is suppressed due to the presence of numerous nuclei [39]. DCPD, on the other hand, is mostly reported to form sheet-like 2D morphologies under slightly acidic conditions [40]. This supports that the reaction medium can indeed change not only the crystals' structures but also their morphologies (**Figure 2a**). Wang et al. explained that at low supersaturation conditions, the energy barrier for crystal formation is high; thus, fewer particles form. Medium

supersaturation results in a lower energy barrier, which leads to the formation of parent and daughter crystals. At higher supersaturation conditions, on the other hand, the energy barrier is so low that nucleation can take place more easily and many crystal branches can form [38]. Calcium phosphate particles can form several crystal morphologies based on the experimental conditions. **Figure 2b** displays the most common crystal morphology of each crystal structure.

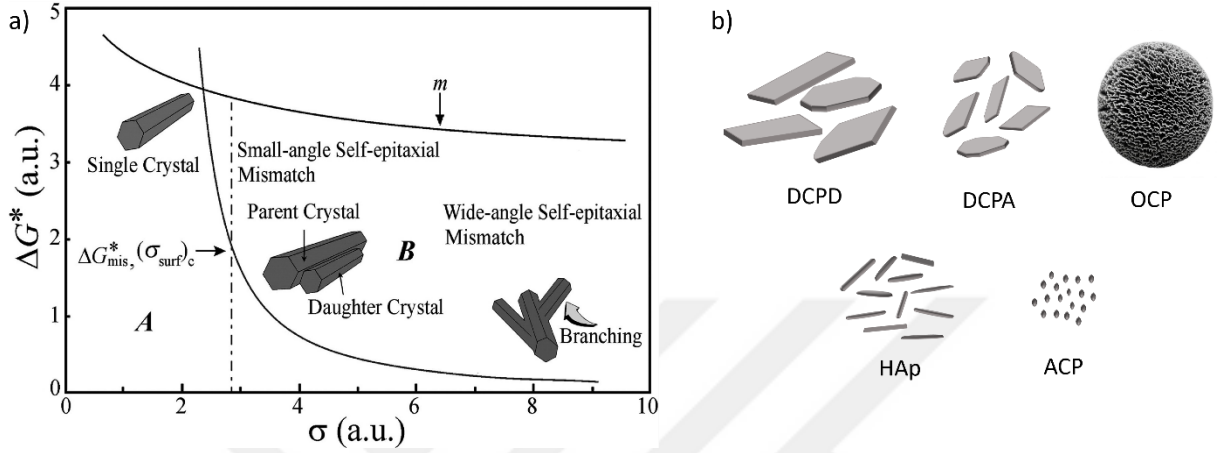


Figure 2. a) Changes in ΔG^* , Gibbs free energy for epitaxial growth of HAp as a function of supersaturation (σ), in which ΔG_{mis}^* , $(\sigma_{\text{surf}})_c$, and m are the activation energy barrier for mismatched secondary crystal, critical surface supersaturation required to form a secondary mismatched nucleation, and mismatch angle between parent and daughter crystals, respectively. Reproduced from Ref [38]. b) The scheme of the most common crystal morphologies of the different CaP phases shown in **Figure 1**.

Development and evolution of different crystal morphologies are important indications and outcomes of the different paths of calcium phosphate biomineralization [22][41][42]. In a synthetic medium, like the hydrogels displayed in this thesis, the formation of crystals in the hydrogel is dominated by diffusion-controlled kinetics [43]. Thus, the crystal structure and morphology are determined by gel-ion interactions and diffusion of ions [19]. The diffusion-controlled process enables greater control over the size and shape of individual crystals compared to other methods [44][45], such as wet precipitation.

1.1.3 Calcium Phosphates: The Effect of Experimental Conditions on the Product Formation

The formation of CaP crystals and their stability are highly sensitive and dependent on the ambient conditions in which they are produced. Various studies have been conducted to mimic the biomineralization of CaP structures under controlled conditions, such as at various pH [42][46] and temperatures [40][47]. The acquired knowledge can be used to design synthetic bioceramic composite materials [20]. The CaP synthesis method has a determinative role in the properties of calcium phosphate precipitates. For example, Anandan et al. discussed that the solid-state reaction method leads to the formation of hydroxyapatite with spherical morphology and superior thermal characteristics. In contrast, the wet chemical precipitation method yields high aggregation of particles with nanoplate-like morphology, and the emulsion method exhibits nanospherical morphology whose properties are dependent on the concentration of phases [48]. (Please see CHAPTER 3 for further information on the wet-chemical precipitation method).

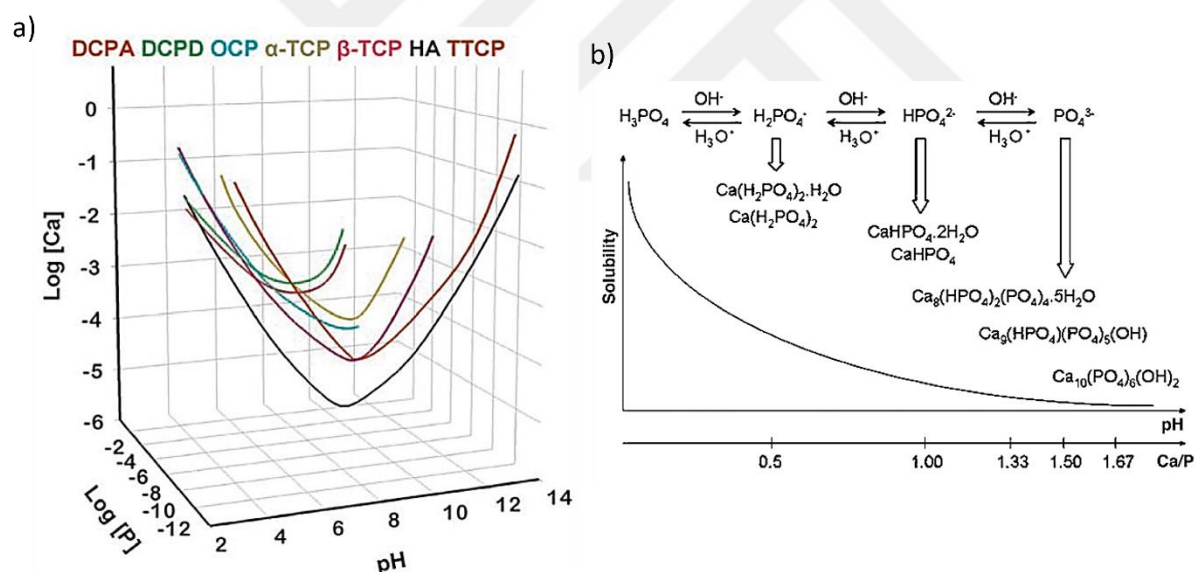


Figure 3. a) Solubility isotherms of several CaP phases as a function of pH and the logarithm of calcium and phosphate concentrations. b) Solubility of different phases depending on pH. The graph also exhibits equilibrium between phosphate ions at different pH values. Figures reproduced from Ref [13].

The pH of the synthesis medium is among the most important factors in determining crystal structure and morphology. Several studies have demonstrated that changes in pH lead to changes in the solubility of CaP [13][47]. Thus, any alterations in pH result in the formation of different crystal structures. **Figure 3a** displays the solubility isotherms of different crystal

structures of calcium phosphates as a function of pH and ion concentrations [13]. The lowest point of each isotherm corresponds to the pH value at which the structure is least soluble and most stable. If any specific combinations of pH and ion concentrations fall above an isotherm, the crystals can form under these conditions. **Figure 3b** shows the changes in the solubility of CaP structures, and the reactions demonstrating the equilibria between phosphoric acid and its deprotonated forms as a function of pH. It is implied in the figure that the type of phosphate predominant in the solution governs the formed product [13].

pH and ion concentrations are not the only parameters determining the crystal structure. Yokoi et al. showed in their work that the precipitate formation temperature in a double-diffusion setup leads to the growth of different crystal structures [47]. At a pH slightly above 7, the dominant phase is DCPD with low intensity peaks of OCP at 4.0 °C. When the temperature is raised to 40 °C, the OCP peak intensity increases with an almost unchanged intensity of DCPD. However, HAp is the only phase when the temperature is elevated to 80 °C [47]. It is a well-established fact that the solubility of many CaP compounds decreases with increasing temperature [47], which results in the insoluble HAp formation at slightly elevated temperatures.

The experimental parameters have an undeniable effect on the particle morphology, too. Changing only the concentration of one of the reactants may lead to changes in both crystal structure and morphology [49]. For instance, higher concentrations of phosphate ions with relatively lower calcium concentrations mainly result in HAp formation [49]. Shin et al. showed that the samples prepared through the sol-gel method are prone to result in varying morphologies based on the concentration of calcium-containing solution and phosphate ions doped in the gel medium. The study demonstrates that the morphology changes with the diffusion direction and reactant concentrations [22]. Another study showed that the cross-linking content in hydrogel resulted in the formation of different crystal morphologies inside and outside of the gel [50].

The incorporation of different ions, e.g., strontium [33], magnesium [51], iron [32], or fluoride [52], into the crystal structure in small amounts affects the properties of formed materials, too [51]. Suguna et al. reported that incorporating fluoride into the CaP crystal structures reduces the size and the number of DCPD crystals [33]. In contrast, magnesium addition leads to the inhibition of DCPD and HAp [51] formation and results in the enrichment of the β -TCP phase. This process mimics magnesium intake in the human body, promoting the

bioactivity of the formed material [51]. On the other hand, the addition of strontium results in the suppression of HAp formation and the promotion of DCPA and DCPD [33]. The average surface area of the strontium-doped HAp crystals is 35% more than that of HAp and shows an improvement in in vitro bioactivity [53].

1.2 Liesegang Patterns (LPs)

For chemical reactions taking place in diffusion-controlled media, the interplay between diffusion and reaction kinetics may lead to the pattern formation in the form of waves [54], stripes [55], and bands [56]. Liesegang patterns (LPs) are a specific type of pattern formation in which a chemical reaction leading to a precipitate is involved. The precipitate is formed in the form of a periodic pattern via simultaneous reaction and diffusion of the co-precipitating ions [54]. The discovery of these structures dates back to 1896, when Raphael E. Liesegang dropped silver nitrate solution into a potassium dichromate solution that was contained in a gel, which formed the silver chromate precipitate patterns [55]. Later, many different precipitation reactions were investigated for their potential to form [55]. These efforts include dispersion of one of the reactants, called as ‘the inner electrolyte’, homogeneously in a medium, mainly in a hydrogel. The other reactant, called ‘the outer electrolyte’, is dissolved in water and allowed to diffuse and react with the former [54]. The concentration of the outer electrolyte must be orders of magnitude higher than that of the inner electrolyte so that the overall process can be designated by only its diffusive flux [54]. A precipitate is formed when (and where) the products of electrolyte concentrations exceed the threshold concentration [55], which is the solubility product of the resultant product. When the ions are consumed by the precipitation reaction, a local depletion of the ions results [54][55]. Therefore, a new precipitate cannot form until some amount of ions diffuses and the threshold is exceeded again. As a result, the next precipitation line forms at a farther distance from the diffusion start compared to the previous one, and likewise, the consecutive lines are formed at longer distances (**Figure 4a**).

When designed in a test tube-like geometry and the diffusion is allowed to occur in the inner electrolyte in the tube, the patterns appear as lines (they are actually discs but still), the patterns are called 1D patterns [56], and the individual precipitation locations are called lines (**Figure 4a**). If the diffusion of the outer electrolyte is allowed from the center of a hydrogel disc (a thin cylinder), the system is called 2D [57], and the pattern is observed as concentric rings (**Figure 4b**). Rarely encountered ones are the 3D LP systems, in which the outer electrolyte diffuses in 3D in a bulk volume hydrogel forming concentric shells [57] of the product (**Figure 4c**).

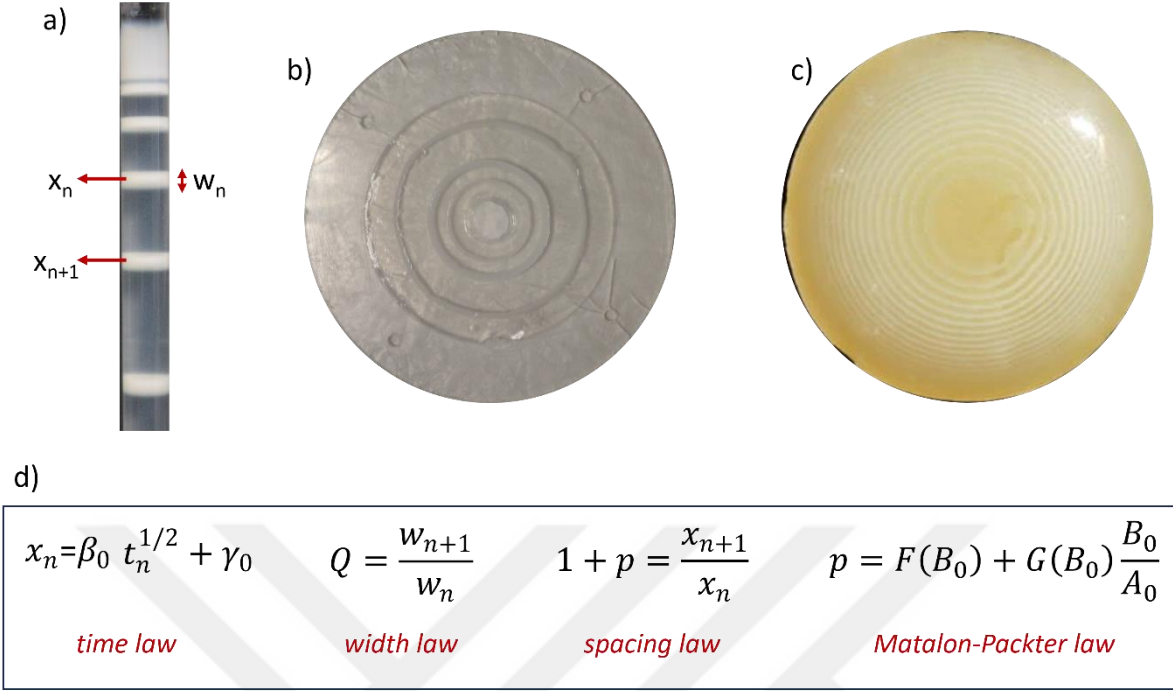


Figure 4. Magnesium hydroxide patterns produced in a) 1D, and b) 2D setups. c) CaP patterns produced in 3D LP setup, reproduced from Ref [62]. d) Empirical rules followed by LPs: time law, width law, spacing law, and Matalon-Packter law. x_n and x_{n+1} represent the position of consecutive bands, whereas w_n and t_n denote the width of the band and time, respectively. A_0 and B_0 are the initial concentrations of the outer and inner electrolytes. $1+p$ is the spacing coefficient, and β_0 and γ_0 are constants.

The regularity in the spacings in LPs led to the establishment of the main empirical rules of the LP systems, forming the characteristics of these systems. These laws are the time, the width, the spacing, and the Matalon-Packter laws [54] (**Figure 4d**). Time law, discovered by Morse and Pierce in 1903, states that the n th band's formation distance is directly related to the square root of time [58]. This law was assumed to resemble the Einstein-Smoluchowski relation for Brownian motion [59]. The width law supposes that the width of a band increases when the distance from the gel-solution interface increases [59]. There is a linearity between the width of the band and its position to the power of α , in which α is a constant depending on the initial concentrations of both electrolytes [55]. The spacing law is the most studied law among all the laws attributed to LPs. According to this law, the distance between bands increases as the band's distance from the outer electrolyte-gel interface increases [55]. The distance ratio between bands is always constant, denoted as the *spacing coefficient* or $1 + p$ [55]. The spacing coefficient is highly sensitive to experimental conditions, and several studies report changes in

the spacing coefficient based on parameters such as type of gel [60], temperature [61], pH [62], and concentrations of inner and outer electrolytes [62]. The Matalon-Packter law was derived from the concentrations of electrolytes. It states that p depends on the initial concentrations of electrolytes. p value declines as a function of increasing outer electrolyte concentration and decreasing inner electrolyte concentration [54].

LP patterns can be observed in several natural systems, like trees [55] and rocks [54]. The initial works on LPs, inspired by these natural patterns, involved the adjustment of several parameters to understand the physical and chemical nature of the process. The recent work on LP is targeting the formation of more complicated systems and products, including metal-organic frameworks (MOFs) [63], gold nanoparticles (AuNPs) [64], Prussian Blue analogs [65], etc. Yet, the most studied and common artificial LPs, e.g., copper (II) chromate [61], cobalt [66] and magnesium [60] hydroxides, silver dichromate [67], and calcium phosphates [56][68], still attract attention for fundamental and applied research.

1.2.1 External Factors Affecting the Pattern Formation

The formation and properties of LPs are highly dependent on inner and outer factors, including gel and electrolyte type/concentration [69], mechanical [57] and electric fields [70], pH [62], temperature [61], the presence of impurities [71], and even light [69] in some cases. In 1939, Isemura published a collective work of different LP systems under varying experimental conditions. This work consisted of the effect of the type and concentration of electrolytes, the concentration of gel, temperature, and pH on the number of bands generated, as well as spacing and secondary banding [69]. Ambrose et al. [72] examined the effect of temperature on the spacing between consecutive bands and found that the spacing of bands decreases with increasing temperature due to the lower solubility of magnesium hydroxide at high temperatures. Our findings also confirm that the spacing coefficient decreases and the number of bands increases with increasing temperature for a calcium phosphate LP system in agarose, in which the solubility of CaP decreases as a function of temperature (**Figure 5**).

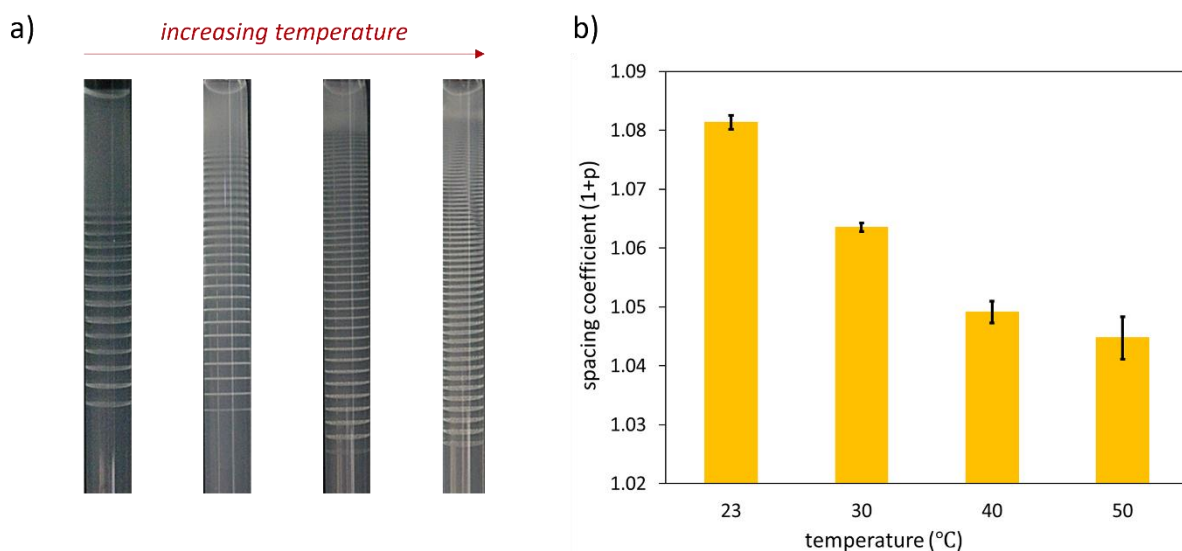


Figure 5. a) CaP LPs in agarose medium ($\text{Na}_2\text{HPO}_4 = 0.015 \text{ M}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O} = 1.5 \text{ M}$) at different temperatures. b) The decrease in the spacing coefficient with increasing LP formation temperature.

In the LP and the related literature, the effect of outer and inner parameters on the chemical system is traditionally reported as the changes in the spacing coefficient, which is depicted as an integral result of all these parameters [71][72]. On the other hand, the variety of the crystal structure and morphology achieved by the reaction-diffusion setups can be used to produce morphology-controlled products using these systems. Therefore, in recent works in the literature, more attention is devoted to the experimental parameters' impact on the microscopic properties of the formed precipitates. Ibrahim et al. demonstrated that the use of single or blend gels not only affects the pattern formation of silver dichromate but also has an influence on the crystal morphology of the products [67]. The previous work of our group, Khan et al. [61], displays how temperature affects both macroscopic and microscopic properties of copper (II) chromate, and that the spacing coefficient and size of precipitated crystals increased with increasing temperature [61]. Another study from the Baytekin group showed that it is possible to control the size of the Co-Fe Prussian Blue analogs using the LP setup. In this work, it was shown that the size control, which is not accessible via solution-based methods due to the very low K_{sp} values of the precipitates and their immediate precipitation upon mixing, is easily obtained in the preparations using the reaction/diffusion method [65]. In this way, it is possible to obtain differently sized products spatially separated in the pattern bands. In other recent studies, the formation of different phases [51] or polymorphs [73] of the precipitate were analyzed. Dimble et al. demonstrated that $\alpha\text{-Co(OH)}_2$ and $\beta\text{-Co(OH)}_2$ polymorphs can separately be formed in an LP system by varying the hydroxide ion sources. An energy storage

application was proposed based on this new synthesis method [73]. Kim et al., on the other hand, showed that the addition of polyethylenimine in the LP system forming ACP regulated the diffusion of ACP and led to continuous band formation, which was hypothesized to enhance bone-tissue regeneration with the product [20].

1.2.2 Biomimetic Control of Pattern Formation

Biomimetics, also known as biomimicry, refers to the field of study that imitates natural biological processes to solve complex problems encountered, e.g., in (bio)material design and drug delivery systems [74]. Through simultaneous diffusion, reaction, and self-organization, biological systems can produce complex patterns, such as Turing patterns on zebra skin [75] or *Ancistrus* fish [75], and patterns of bacterial colonies [76]. Several Liesegang patterns or Liesegang-like structures have also been shown to exhibit biomimetic pattern formation. Klug et al. demonstrated that fossil tissues of the Mesozoic coleoids possess silification rings, supporting the natural formation of non-calcium phosphate patterns [77]. LPs serve as an ideal pattern set for biomimetic patterning since they possess periodicity [78]. Especially, biominerals such as calcium carbonates [79] and phosphates [56] can be formed in the hydrogel media via a controlled mineralization process. Another biomineral-forming biomimetic LP system is the one producing calcium oxalate from the relevant ions through precipitation in a gel. Thomas et al. produced calcium oxalate LPs in agar medium with a double-diffusion method. The study examined the morphological control of calcium oxalates to mimic kidney or urinary calculi. They found out that pH had a determining role in the formation of different morphologies. It was concluded that this study might help to investigate the multi-step processes in stone formation [80].

1.3 The Role of Gel Medium in LPs and Biomineralization

A gel medium is preferred almost exclusively for generating synthetic LPs to provide the necessary aqueous environment for the reaction and to cease convection [54] for a solely diffusion/reaction-controlled kinetics [43] of the overall process. On the other hand, chemical interactions of ions with the gel medium have a determinative role in both macroscopic and microscopic properties of patterns. Gel polymer concentration is one factor that affects the extent of these interactions. For example, Itatani et al. [78] concluded that the periodicity of copper (II) chromate LPs could be programmable by adjusting gel concentration. The authors claim that LP-like patterns in nature exhibit complex periodicity rather than regular type periodicity [78], in which the distance between two consecutive bands increases as a function of the distance from the gel-solution interface. In their work, they successfully obtain complex

periodicity by using a gel concentration gradient with varying concentrations. Using the same chemical reaction, our group [81] showed that using a gel concentration gradient and various macroscopic geometrical shapes between concentration boundaries, the periodicity and the spacing coefficient of the LPs can be altered drastically. The gel concentration gradient also led to the formation of varying crystal morphology in patterns across a gradient boundary. It was concluded that such a system could be programmable in distinct dimensions to design complex patterns of matter [81].

Another way of affecting the LPs forming in a hydrogel is to add impurities to the hydrogel or make a blend with another gel. Interestingly, the first reports on LP formation in such blend gels were published almost seven decades later than the studies on other parameters. Lagzi showed that using an agarose gelatin blend, in which gelatin is added as an impurity, results in spacing coefficient changes of copper (II) chromate precipitation bands [71]. Lagzi et al. also showed that silver dichromate LPs cannot be formed in agarose hydrogel, whereas the use of gelatin results in successful pattern formation. The mixture of both gels may or may not produce Liesegang banding based on the concentrations of each gel [82]. The results support the idea that the gel medium and its concentration in the blend are crucial parameters for obtaining patterns.

In a biomineralization process, a gel medium provides an organic scaffold to promote nucleation and particle growth of inorganic materials, which shapes the particles and determines the direction of precipitate formation. Hydrogels are highly preferred organic matrices in biomineralization studies due to their properties similar to the extracellular matrix [83]. They also control mass transfer and determine the location and number of nucleation centers [43]. In the case of CaP biominerals, collagen [84], gelatin [19], and chitin [84] are used. Some other polymers have also chemical groups capable of binding to calcium and phosphate ions [85] and acting as possible nucleation centers (**Figure 6**). For example, it is known that carboxylate groups of gelatin can interact with calcium ions and act as a nucleation site [19]. Zhang et al. [62] explained the differences between the spacing coefficients of the patterns formed at various pH values by the deprotonation extent of the carboxylates of the gelatin at the different pHs. The presence of carboxylate groups was also hypothesized to reduce the activation energy for nucleation of CaP [86]. On the other hand, if agarose – a relatively inert gel with neutral structure [87] – is used as a matrix, a less significant effect can be expected. Still, the absence

of strong chemical interactions does not imply that inert matrices do not have any role in crystal formation. Agarose acts as a structural scaffold that controls the diffusion of ions [22].

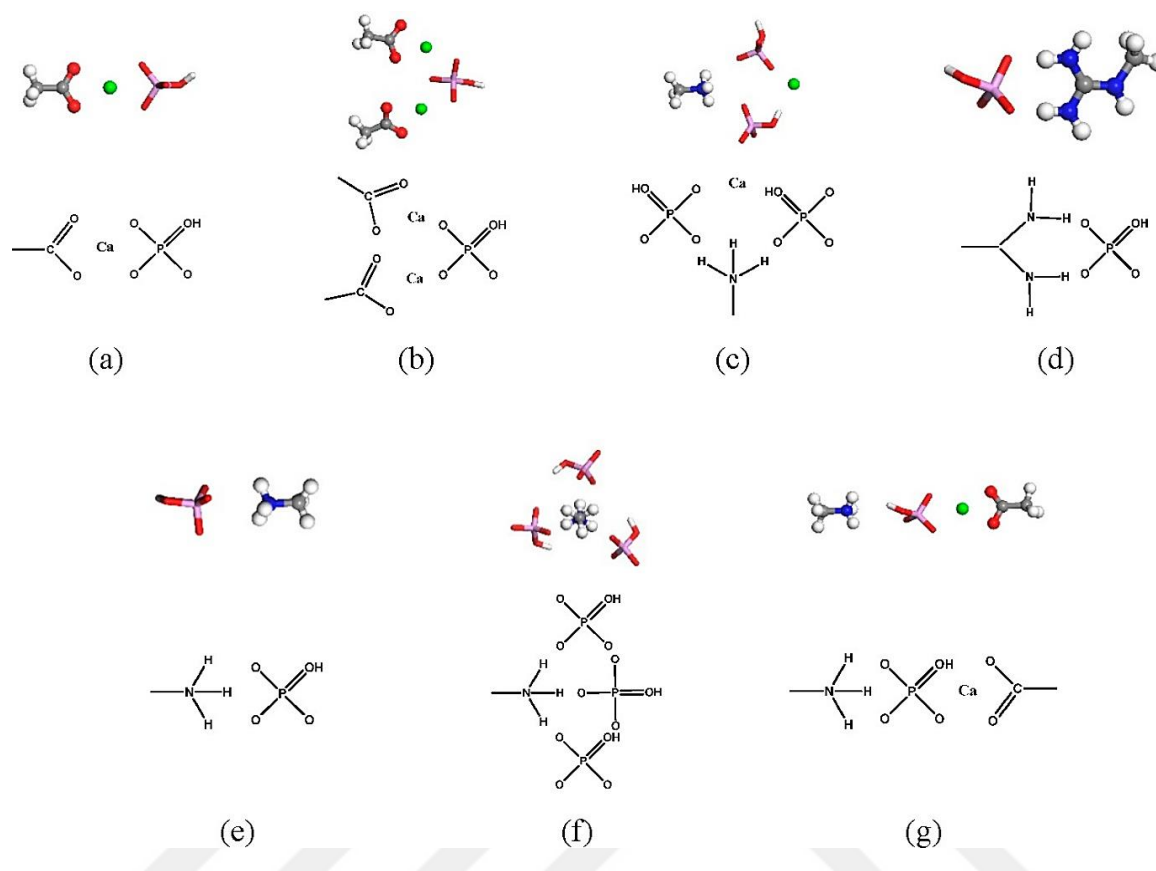


Figure 6. Possible chemical interactions between calcium ions, phosphate, and functional groups of polyamides that can act as nucleation centers, reproduced from Ref [85].

1.4 Calcium Phosphate Liesegang Patterns

CaP LPs have been amongst the most investigated LPs in literature [88][89][90]. The earlier practices on these systems mostly focused on understanding why and how calcium phosphates were produced inhomogeneously [88] or in complex patterns [89]. Complex patterning in these systems was explained by the Ostwald ripening mechanism. According to this explanation, a depletion of small particles leads to rapidly growing aggregates and leaves a broad plateau behind [89].

One of the earliest studies on CaP LP formation advocated that the composition and crystal structures of products forming on patterns should vary in different regions; however, the experimental results concluded that the crystals had identical structures [88]. The other studies concerning CaP Liesegang pattern formation focus on either the synthesis of specific phases like hydroxyapatite [34] or the effect of experimental conditions on the forming products [49]. For example, a study reported that the spacing coefficient of products increased with an increase

in pH [91]. On the other hand, the presence of a magnetic field was shown to enhance the ordering of crystals as well as to increase the length and width of growth [92]. Jo et al. showed that the crystal growth of calcium phosphates in a gelatin medium was asymmetric, and the crystal density of bands at different regions was strikingly different from one another [68]. Boskey analyzed the effect of the inclusion of collagen and non-collagenous proteins (e.g., proteoglycans) on precipitate formation. The study revealed that type I fibrillar collagen did not promote CaP formation, even though it decreased the time of first precipitate formation. Proteoglycans, on the other hand, were found to inhibit HAp formation [21].

The inclusion of other compounds has also proven to form a variety of crystal morphologies in addition to the formation of different structures. Cho et al. used PAA additives in a gelatin medium to prove that particle morphologies can be altered by utilizing blends of different hydrogels [56]. The addition of macromolecules has been examined extensively in CaP LPs. Salve et al. investigated the effect of sucrose and glycine on CaP pattern formation in agar gel and demonstrated that the presence of macromolecules significantly alters crystallinity, morphology, and the spacing coefficient [93]. A study focusing on biphasic calcium phosphates (BCP) concluded that magnesium-doping to the outer electrolyte solution changed the morphology from anisotropic fibers to tiny spherical particles. This new system was assumed to enhance bioactivity, whereas mechanical properties such as hardness and elastic modulus decreased [51]. Doping the outer electrolyte solution with an iron-containing molecule also leads to variations in crystal morphology, which is hypothesized to have an application in protein adsorption and studies on biomineralization [94]. In another work on CaP LPs, Deng et al. confirmed that using a hybrid agar-gelatin hydrogel medium led to the formation of varying morphologies than that of single gel systems at different temperatures [19].

Most recent studies on CaP LPs focus on the potential application of such systems. Choi et al. concluded that LP formation in biomineralized gels could promote non-linear elasticity [95]. They observed that the Liesegang-patterned complex exhibited stress relaxation, which is a property ascribed to cartilage. It was claimed to have a higher endurance to deformation thanks to stress relaxation. In another recent work, Wang et al. showed that when biomineralized hydrogels form a biomimetic hierarchical structure similar to LP-like patterns, they were prone to exhibit significant increases in migration on osteogenic differentiation of bone-related stem cells. The study also includes *in vivo* experiments for bone regeneration, and the Liesegang patterned hydrogels have been demonstrated to promote bone regeneration [90].

LP formation is not the only route to obtain CaP precipitates in a gel medium in a controlled way. Some double-diffusion [50] and single-diffusion [49] experiments have also been conducted to form CaP precipitates with different morphologies in gel media. **Table 1** summarizes CaP prepared under different experimental conditions and the resulting morphologies.

Table 1. Literature summary of CaP biominerals produced in various gel media. The table shows varying experimental conditions and synthesis methods that lead to the formation of different crystal morphologies.

	Gel	Inner	Outer	pH & T	Morphology	Source
Double-diffusion experiments	Agar	$(\text{NH}_4)_2\text{HPO}_4$	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	7.6 – 28 °C	needle-like, irregularly shaped aggregates	[25]
	PEGMEM + DMAEM	Na_2HPO_4	CaCl_2		round-shaped crystals and aggregates	[50]
	Gelatin	Na_2HPO_4	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	7.4 – 25 °C	sphere-like and flakes	[52]
Single-diffusion experiments	Silica	H_3PO_4	CaCl_2	10.5	spherule	[34]
	PAM	$(\text{NH}_4)_2\text{HPO}_4$	$\text{Ca}(\text{NO}_3)_2$	-	wool-ball-like	[86]
	Silica	H_3PO_4	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	5-11 – 4.0 °C, 40 °C and 80 °C	irregular-shaped, rod-shaped, spherulite, ribbon-like (pH and temperature dependent)	[47]
	Agarose	Na_2HPO_4	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	RT	flower-like, plate-like, ribbon-like, granules, (concentration-dependent)	[22]
	PAM	$(\text{NH}_4)_2\text{HPO}_4$	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	7.7-7.9 – 40 °C	plate-like, spherulite, granular (concentration-dependent)	[49]
Single-diffusion (LP) experiments	agar- gelatin	$\text{Ca}(\text{NO}_3)_2$	Na_2HPO_4	10-37 °C and 65 °C	rod-like (37 °C) needle-like (65 °C)	[19]
	Silica	Na_2HPO_4	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	7.4 – 37 °	spherulites with platy crystals (37 °C), platy crystals (27°C)	[53] [96] [97]
	Silica	H_3PO_4	CaCl_2	6.0	needle-like	[33]
	Gelatin	Na_2HPO_4	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	24°C	ribbon-like	[68]
	Gelatin + PAA	Na_2HPO_4	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	RT	granules	[56]
	Gelatin	Na_2HPO_4	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	RT	plate-like	[20]

1.5 The Purpose of the Work

As briefly depicted in the above sections, many studies have been conducted to understand and control the formation of Liesegang patterns for over a century. Among these, the studies concerning crystal morphology and crystal structure are still rare. On the contrary, studies focusing on biomineralization and bioceramic composite materials are rapidly growing. Interestingly, the crystal structures or morphologies of CaP LPs are not examined thoroughly despite the abundance of these biomineralization studies. Considering the importance of biominerals for hard tissue formation and adaptation, as well as for biomaterial design, it is crucial to control the structure and morphology of crystals precipitated in organic matrices, as in the case of the natural biomineralization process. As depicted above, numerous studies have already contributed to both Liesegang pattern formation and the biomineralization of CaP precipitates. Nevertheless, there are still a number of challenges and undiscovered realms of the CaP and LP formation. Some of the challenges and undiscovered parameters include:

- The use of simple laboratory settings to mimic the natural formation of the minerals, which undermines the complexity of scaffolds and the natural biomineralization process.
- The lack of studies considering both protein and polysaccharide components of the extracellular matrix.
- The rarity of practical applications of CaP LPs, even though properties of the precipitate can be controlled with slight arrangements in experimental conditions such as gel composition, pH, temperature, and direction of diffusion.
- The use of crosslinking agents, which mostly leads to cytotoxic effects.

In this study, agarose-gelatin blends are utilized to form Liesegang bands of calcium phosphates. Gelatin provides a scaffold similar to the extracellular matrix of bone and possibly promotes the formation of nucleation sites due to the abundance of functional groups. In contrast, agarose acts as a relatively inert matrix providing structural support. Thus, the aims of this work are:

- Effectively controlling the particle morphology by adjusting only the gel composition.
- Exploring the effect of hydrogel blending on the biomineralization process. Blends of a polyamide and a polysaccharide gel resemble the extracellular matrix. Therefore, an understanding of CaP formation in such a blend can provide useful information about the mechanisms of natural biomineralization.

- Understanding the mineralization process at different stages of spatiotemporal pattern formation, as well as answering how crystals form and adapt at different ambient conditions.

Our experimental results yield a successful formation of varying particle morphologies within a single Liesegang band in a repeating manner. These hierarchical within-band variations across different generations of bands are observed in the literature for the first time. The biominerals in natural bone structure are also formed hierarchically [20]. Therefore, any insight into such organization levels, such as the one displayed in this work, helps to understand the underlying mechanisms of biomineral formation and is invaluable in the design of bioceramic materials.



CHAPTER 2

2. Materials and Methods

2.1. Materials

Gelatin (Zag Kimya), agarose (Isolab Chemicals), magnesium sulfate (MgSO_4 , AFG bioscience, anhydrous), ammonium hydroxide (NH_4OH , Sigma Aldrich 25-30%), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma Aldrich, 99%), sodium phosphate dibasic (Na_2HPO_4 , Carlo Erba, anhydrous), Type I collagen (MSA Kozmetik), Type I&III collagen (Naturagen), sodium hydroxide (Merck, pellet), nitric acid (Sigma Aldrich, 65%).

All chemicals were used as received and without further purification.

Parafilm® was used without any chemical modification.

2.2. Choosing the Gel Blends as the Media for CaP formation

As summarized in the previous chapter, the formation of LPs and the crystal structures of particles in the LP bands are dependent on several parameters, some of which are the gel and electrolyte type and concentration, the presence of crosslinking agents and/or substitute ions, pH, and temperature. Although there are many candidates of polymers that gelate under certain conditions, we chose two of the simplest and most available polymers, agarose and gelatin, as the polysaccharide and the polyamide that resemble the extracellular matrix and tissues. Agarose is a mechanically stronger hydrogel [87] than gelatin and serves as a support, while gelatin provides the chemical groups for nucleation and growth of the crystals [19]. The reports on the calcium phosphate LP systems, displaying that CaP is prone to having inhomogeneous and asymmetric periodic precipitate formation [68] and that the use of different additives may lead to changes in crystal morphology [20][56], motivated us to use the varying compositions of these gels in the blend to try to obtain different products. So far, there are not many studies focusing on the spatiotemporal formation of CaP LPs in a polysaccharide-protein blend [19]. A few studies use a blend of both polymers to form CaP LPs [19], but there are no reports of a hierarchical within-band variety of CaP.

The concentration of the gel blend is chosen to have the total polymer concentration at 5.0% (w/v). This concentration is a moderate gel concentration; which is low enough to enable the diffusion of the ions in a manageable time (a few days) to obtain the LPs, and high enough to keep the gel form to retrieve the products for analysis from the LP bands, once they form.

Changing only the ratio of the polymers in the blend and keeping the total amount of polymer constant helped us to maintain similar mechanical integrity of the samples prepared throughout the varying-concentration blends.

2.3 Gel Preparation

2.3.1 Preparation of the Agarose-Gelatin Blend Blank Samples

Blank samples of gel blends were prepared to verify whether blending leads to some unspecific chemical interactions between the gels and if the blend formed homogeneously. Agarose and gelatin polymers were separately dissolved in distilled water in different proportions. When each polymer was fully dissolved, the gelatin solution was added dropwise to the agarose solution. The mixture solution was transferred to a glass Pasteur pipette (5.0 mm diameter and 10 cm length) with a 1.0 mL syringe. Three-fourths of the pipette was filled with the mixture solution. 3 replicate samples for each batch blend ratio were prepared to determine the homogeneity of the samples. The same procedure was repeated, but this time gelatin was doped with 0.020 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (concentration after blending). After dissolving gelatin with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, it was added to the agarose solution for blending. The samples were aged for 3 days. Following this, the samples were washed 3 times (over a period of 3 hours) with 10-15 mL of deionized water each time.

2.3.2 Preparation of the Gelatin and Agarose (control) Samples and the Agarose-Gelatin Blend Samples for CaP LP formation

5.0 % (w/v) Gelatin samples (control). 0.75 g of gelatin and 0.044 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were dissolved with vigorous mixing in 15 mL of distilled water at 90 °C to obtain 5.0% (w/v) gelatin and 0.020 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in the solution. The solution was transferred to a glass Pasteur pipette (5.0 mm diameter and 10 cm length) with a syringe. Three-fourths of the pipette was filled with polymer solution. (The rest of the pipette volume was used to accommodate the outer electrolyte solution.) 5 replicate samples were prepared using the same method. The samples were left to gelate overnight before further treatment. After complete gelation, the walls of the pipettes were cleaned of any gel residues. 0.60-0.70 mL of the outer electrolyte solution, 0.50 M Na_2HPO_4 , was added on top of the gel containing the inner electrolyte. The tops of the pipettes were covered with parafilm to prevent the solution from evaporating. The samples were left at room temperature (set to 25 °C) for 4 days for pattern formation.

5.0 % (w/v) Agarose samples (control). 0.75 g of agarose and 0.044 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 15 mL of water were heated in a microwave until a transparent solution was obtained. After

this step, the same procedure was applied to the agarose samples as the one depicted for gelatin control samples above, except for the gelation time of 30 minutes.

x % Agarose – (5.0-x)% Gelatin blend samples. To produce blend samples, 0.041 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and gelatin (the amount varied based on the individual polymer concentrations in the blend, as in **Table 2**) were dissolved in 7.0 mL of distilled water at 90 °C with vigorous mixing. 7.0 mL of distilled water was added to the agarose (the amount varying based on individual polymer in the blend, as in **Table 2**). The mixture is heated on a hot plate and then transferred to a microwave to dissolve the agarose completely. After complete dissolution of both polymers, gelatin solution containing the inner electrolyte was added to the agarose solution on a heater dropwise with continuous stirring to prevent the phase separation of hydrogels. The polymer mixture solution was stirred for 1 to 2 minutes more after the addition was completed. The solution was transferred to a glass Pasteur pipette (5.0 mm diameter and 10 cm length) with a syringe. Three-fourths of the pipette was filled with polymer solution. (The rest of the pipette volume was used to accommodate the outer electrolyte solution.) 5 replicate samples were prepared using the same method. The samples were left to gelate before further treatment. 0.60-0.70 mL of the outer electrolyte solution, 0.50 M Na_2HPO_4 , was added on top of the gel containing the inner electrolyte. The tops of the pipettes were covered with parafilm to prevent the solution from evaporating. The samples were then left at room temperature (set to 25 °C) for 4 days for pattern formation (**Figure 7**).

Table 2. The amounts of polymers used to prepare corresponding blend concentrations

<i>Polymer blend ratio Agarose/Gelatin</i>	<i>amount of gelatin polymer used (g)</i>	<i>amount of agarose polymer used (g)</i>
5.0 G	0.70	0
1.0 A / 4.0 G	0.56	0.14
2.0 A / 3.0 G	0.42	0.28
2.5 A / 2.5 G	0.35	0.35
3.0 A / 2.0 G	0.28	0.42
4.0 A / 1.0 G	0.14	0.56
5.0 A	0	0.70

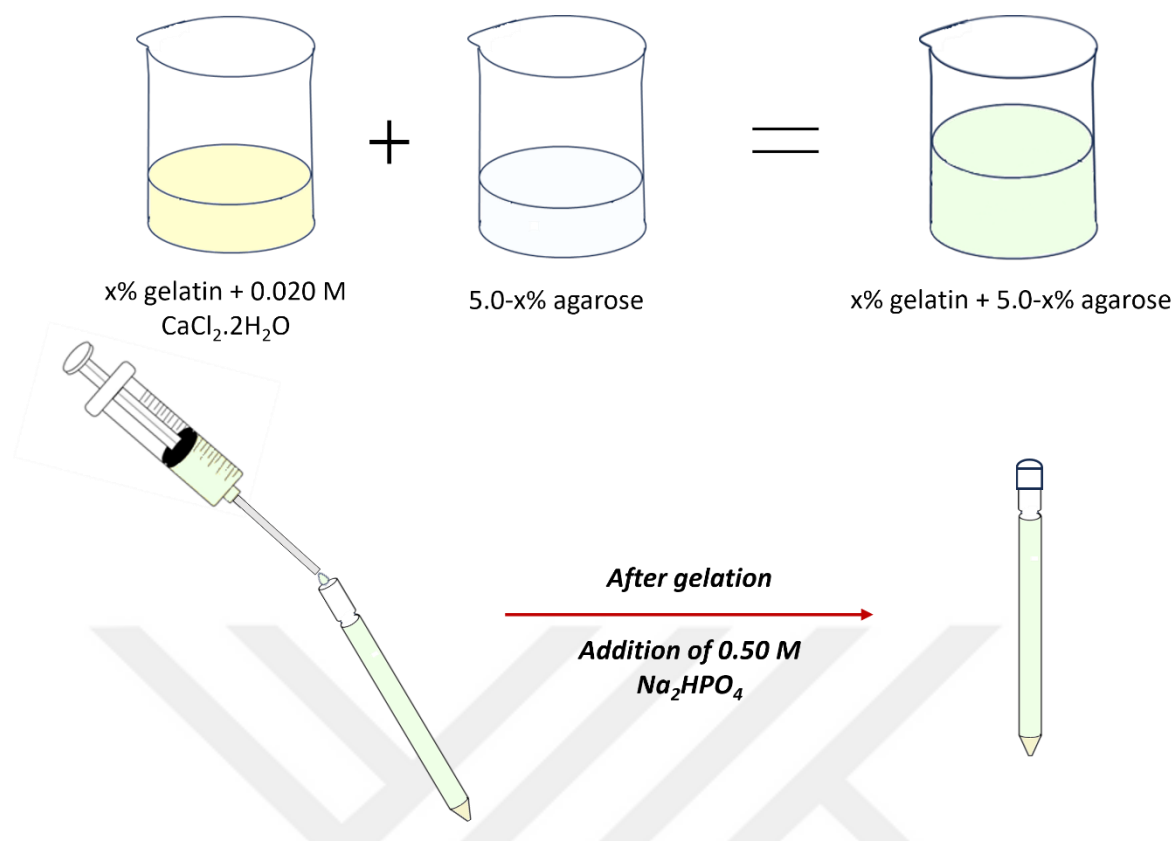


Figure 7. The illustration of the experimental setup for the preparation of the gel blends and LP patterns. The gels are dissolved separately, while the inner electrolyte ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.020 M) was added to the gelatin solution. Gelatin/inner electrolyte solution was then added dropwise to agarose solution. After mixing, the final solution was transferred to a glass Pasteur pipette. The solution was allowed to gelate, and after the gel formed, the outer electrolyte (Na_2HPO_4 (aq), 0.50 M) was added into the pipette, to the top of the gel to start the LP formation.

Figure 8 shows a representative sample, samples before and after the outer electrolyte addition, and patterned single and blend hydrogels.

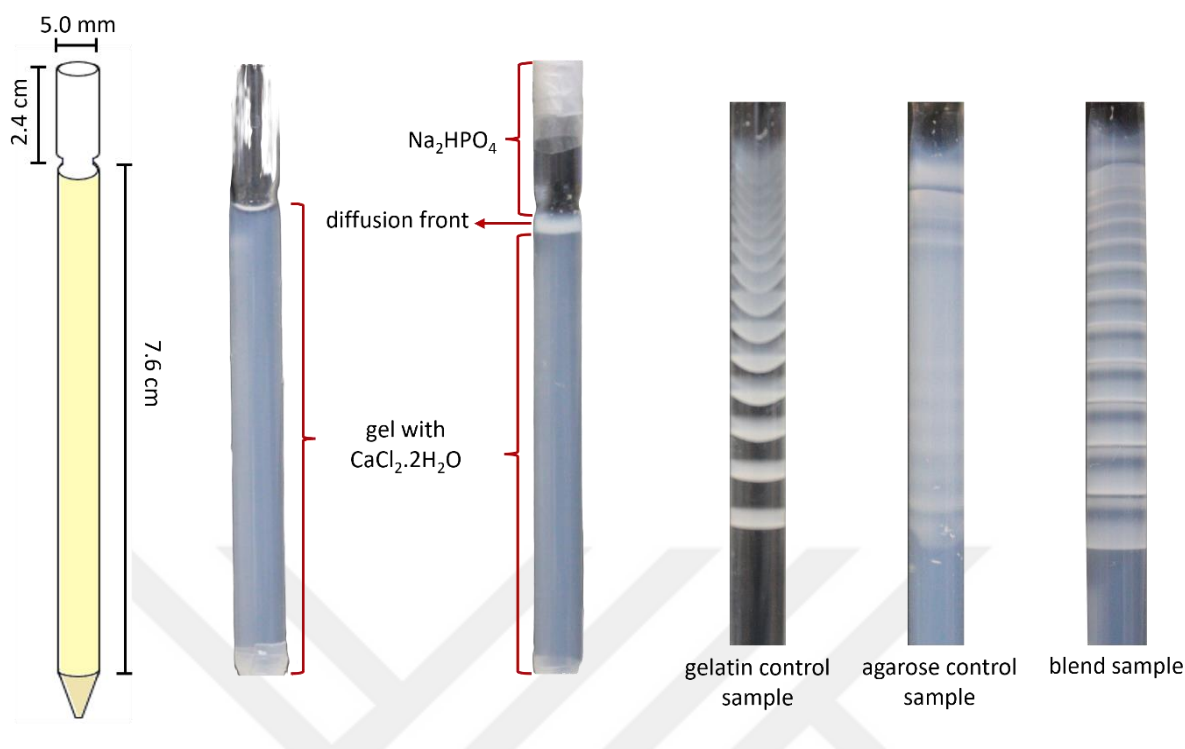


Figure 8. The samples before and after outer electrolyte addition (left). CaP LPs formed in gelatin, agarose, and an agarose/gelatin blend. (Polymers and the polymer blend = 5.0% (w/v))

2.3.3 Preparation of the Gelatin-Collagen and Agarose-Collagen Blend Samples for CaP LP formation

8.0 % (w/v) Gelatin samples (control). 0.80 g of gelatin and 0.029 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were dissolved in 10 mL of distilled water to produce an 8.0% (w/v) gelatin solution with 0.020 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ as the inner electrolyte. The solution was transferred to Pasteur pipettes and left to gelate overnight as described in the previous section. After gelation, the outer electrolyte solution was poured on top of the pipette, and the patterns were left to form for 4 days.

6.0 % Gelatin – 2.0 % Collagen blend samples. Gelatin-collagen blend samples were prepared identically to the agarose-gelatin blends described in the previous section, except for the concentration of the individual polymers. The concentration of gelatin was set to 6.0% (w/v) while the collagen concentration was 2.0% (w/v). The samples were prepared with 2 different collagen types: collagen type I (marine type), and collagen type I&III. Both gelatin and collagen were added to the inner electrolyte in different sets of samples separately to observe the effect of functional groups present in the amino acid residues of the chemicals.

4.0 % (w/v) Agarose samples (control). 0.40 g of agarose and 0.029 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were dissolved in 10 mL of distilled water to produce a 4.0% (w/v) agarose solution doped with 0.020 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ as the inner electrolyte. The solution was transferred to Pasteur pipettes and gelled as described in the previous section. When gelation was completed, the outer electrolyte solution was introduced. The total pattern formation time was 4 days.

3.0 % Agarose – 1.0 % Collagen blend samples. Agarose-collagen blend samples were prepared identically to the gelatin-collagen blends described in the previous section, except for the concentration of individual polymers. The concentration of agarose was set to 3.0% (w/v) while the collagen concentration was 1.0% (w/v). The samples were prepared with 2 different collagen types: collagen type I (marine type), and collagen type I&III.

2.3.4 Magnesium Hydroxide Control and Blend Samples

5.0 % (w/v) Gelatin samples (control). 0.50 g of gelatin and 0.24 g of MgSO_4 were dissolved in 10 mL of distilled water with a heater at 530 rpm and 90 °C to obtain 5.0% (w/v) and 0.20 M concentrations, respectively. The same procedure for the preparation of CaP gelatin control samples (described in the previous section) was followed for the gel preparation. After complete gelation, the upper part of the Pasteur pipette was filled with 0.60 to 0.70 mL of concentrated (6.93 M) NH_4OH . The top of the Pasteur pipette was covered with parafilm, and the samples were left at room temperature for pattern formation. The total pattern formation time was 3 days.

5.0 % (w/v) Agarose samples (control) and the Agarose-Gelatin blend samples. Agarose control samples and blend samples were prepared using the same method described for CaP samples, except for the identity and the concentrations of inner and outer electrolytes, which are mentioned in the above paragraph.

2.4 Experiments for CaP LP formation at varying Temperatures

Temperature-dependent experiments were run at 4.0° C, room temperature (25° C), and 37° C. The samples were prepared identically (Please refer to Section 2.3.2) and put in a refrigerator or a home-made temperature controlling device. The setup of the device is shown in **Figure 9**. The system contains a Peltier element controlled by Arduino Uno. The Peltier used was CP2-127-10-L-EP-W4.5 (Thermoelectric Cooler with 64975-503 MFG part number). It is a 62 mm x 62 mm element with a maximum voltage of 13.9 V and a maximum current of 9.3 A. The element provides both cooling and heating based on the programming. The Peltier was sandwiched between a heat sink (HYPER TX3 EVO) and an aluminum plate. A small amount

of thermal paste was applied to both sides of the Peltier to protect the setup and increase thermal conduction. A K-type thermocouple sensor was used, which works between -20 to 80 °C (temperature sensitivity of 0.25 °C). One sensor end was placed between the aluminum plate and another smaller aluminum plate, while the other connected to Arduino Uno with the MAX

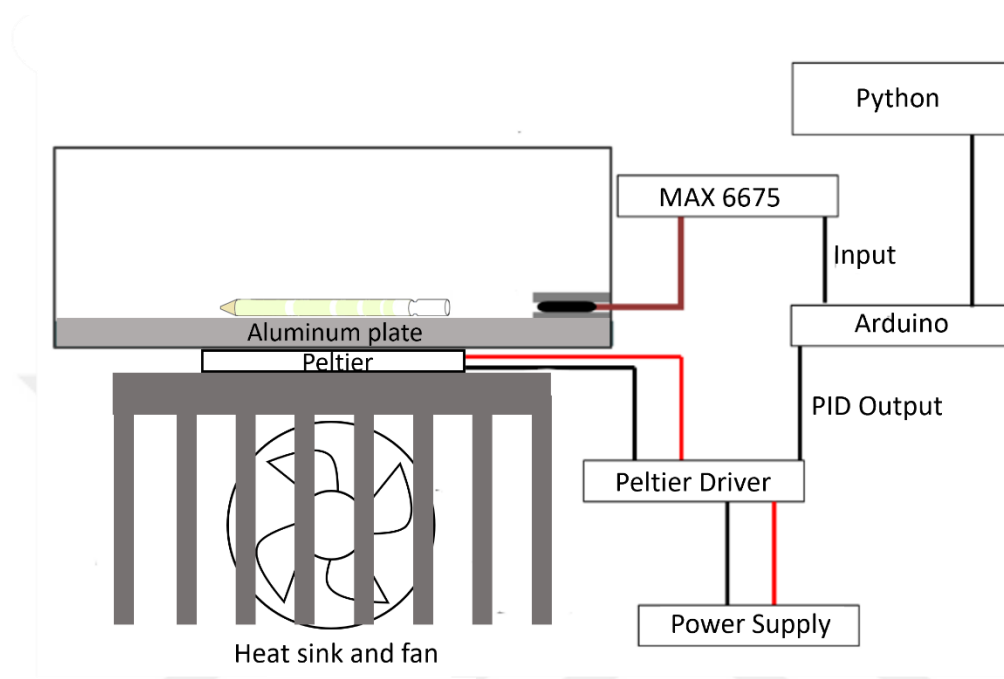


Figure 9. The setup of the home-made temperature control device consisting of a Peltier element and an Arduino Uno.

6675 chip. A Spyder Python code, connected to the Arduino Uno, was used to program the desired temperature. Peltier Driver (BTS7960B 40 Amp DC Motor Driver) gets the output of MAX 6675 (after PID controller loop in Arduino) and drives the Peltier.

2.5 Experiments for CaP LP formation under varying pH conditions

As the crystallization and phase formation of CaP biominerals are highly dependent on the pH of the gel environment, the formation of CaP in gels can be examined under controlled pH conditions, in which the pH of only the outer electrolyte solution, only the gel environment, or both are controlled. In our experiments, we prepared samples in which the gel and the outer electrolyte are set to some pH values by acidifying or basifying the media with 4.5 M nitric acid and 3.0 M sodium hydroxide. pH papers were used to check the approximate pH of the outer electrolyte and the polymer solutions before gelation.

Acidifying or basifying the outer electrolyte. The pH of a 0.50 M sodium hydrogen phosphate outer electrolyte solution was determined as 9.0. Either acid or base was added with

a syringe dropwise to a 0.50 M solution while the solution was continuously stirred with a PTFE-coated magnetic stirring bar. The pH of the solution was continuously checked with pH papers during the process. After mixing the solution for at least a minute, pH 10.5, pH 9.0, pH 7.0, and pH 5.5 outer electrolytes were prepared and loaded on top of the gel for pattern formation.

Table 3. Formation of LPs under different outer electrolyte and gel pH conditions. The “-” sign means that the reaction did not produce a visible precipitate, while the “+” sign refers to systems producing LPs. When the gel pH was set to 4.0, patterns formed relatively weakly compared to other systems. ppt and cts are short-hand notations for ‘random precipitate formation’ and ‘continuous band formation’, respectively.

Outer electrolyte pH	Gel pH = 4.0	Gel pH = 5.5	Gel pH = 12
5.5	(-)	(ppt)	(cts)
7.0	(weakly)	(+)	(cts)
9.0	(relatively weakly)	(+)	(cts)
10.5	(relatively weakly)	(+)	(cts)

Acidifying or basifying the blend gels. The pH of the as-prepared blend of 2.0% (w/v) gelatin and 3.0% (w/v) agarose, containing 0.020 M of calcium chloride dihydrate, was determined to be between 5.0 and 6.0. The gel pH values were set to 4.0, 5.5, and 12. The acidification and basification of the gels were performed before blending and without inner electrolyte addition. (Therefore, the predetermined pH values listed above in **Table 3** belong to the gelatin solution only.) 0.28 g of gelatin was dissolved in 6.0 mL of distilled water. The pH of this solution was recorded as 5.5. Either the acid or the base was added dropwise with continuous stirring and heating until the desired pH was obtained. Then, 0.041 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved in 1.0 mL of distilled water and added to the gelatin solution. The gel-inner electrolyte solution was continuously stirred until the solution was homogeneous. This solution was added dropwise to previously dissolved agarose gel (0.42 g of agarose and 7.0 mL of distilled water) with continuous stirring. The blend solution was transferred to the Pasteur pipettes and left to gelate at room temperature (as described in the above sections for other samples). 5 replicate samples were prepared using the same method. After complete gelation, 0.60-0.70 mL of the outer electrolyte solution, 0.50 M Na_2HPO_4 , was added on top of the gel containing the inner electrolyte. The tops of the pipettes were covered with parafilm to prevent the solution from evaporating. The samples were left at room temperature (set to 22 °C) for 4 days for pattern formation.

2.6 Solution Synthesis of CaP

0.29 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved in 100 mL of deionized water to obtain 0.020 M of stock solution. 1.4 g, 0.85 g, 0.57 g, 0.28 g, 0.14 g, and 0.057 g of Na_2HPO_4 were dissolved each in 20 mL of deionized water to obtain 0.50 M, 0.30 M, 0.20 M, 0.10 M, 0.025 M, and 0.020 M of solutions, respectively. 20 mL of calcium chloride dihydrate solutions were slowly added to equal volume of the prepared sodium hydrogen phosphate solutions with continuous stirring at 200 rpm and room temperature. The top of the beaker containing the precipitate solution was covered with parafilm, and the solution was stirred overnight (at least for 15 hours) at the same ambient conditions. The samples were filtered using vacuum filtration using a 2.5 μm pore size filter paper. The precipitates were washed three times with 60 mL of deionized water and 60 mL of ethanol. The samples were dried under vacuum.

2.7 Ion – Gel Interaction Experiments

Two sets of samples were prepared for IR spectral and SEM analyses to show the effect of the functional groups on the gel on the ion binding and CaP formation. In the first set, 0.10 g of agarose was dissolved in 5.0 mL of distilled water, and 0.029 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.0018 g of gelatin were dissolved in 5.0 mL of distilled water together. After both gels had completely dissolved, gelatin solution was added dropwise to the agarose solution. The solution was stirred for an additional 1 to 2 minutes before gelation. The final concentrations of agarose and gelatin were 1.0% (w/v) and 0.018% (w/v), respectively. 0.10 g of agarose and 0.029 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were dissolved in 10 mL of distilled water to prepare agarose control samples.

In the second set, 1.0% (w/v) agarose and 5.0% (w/v) gelatin gels were also prepared as control samples, without adding ions. Gelatin solution was also added to 0.050 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, whereas 0.010 M and 0.10 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were added to agarose in preparation for these samples. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solutions were directly doped in hydrogels. 0.50 M of Na_2HPO_4 solutions were also allowed to diffuse in some of the control samples to understand how binding (if any) occurs in experimental conditions. All the samples were let to age for 4 days. Following this, the samples were washed with 6.0 – 7.0 mL of deionized water three times, each time for 10 minutes, to avoid affecting the ion-gel interaction. Small sections of gel samples were separated and dried under vacuum for IR spectroscopy.

2.8 Characterization and Analysis

2.8.1 Characterization

Sample preparation for analysis. The LP containing gels were carefully taken out of the Pasteur pipettes. The gels were then washed to remove excess/spectator ions. The parts of the sample containing patterns were separated from the rest of the sample and washed four times for 4 hours with 50 mL of deionized water. The individual bands were then cut with a steel blade, collected separately, and dried for analysis as depicted for each type of analysis below.

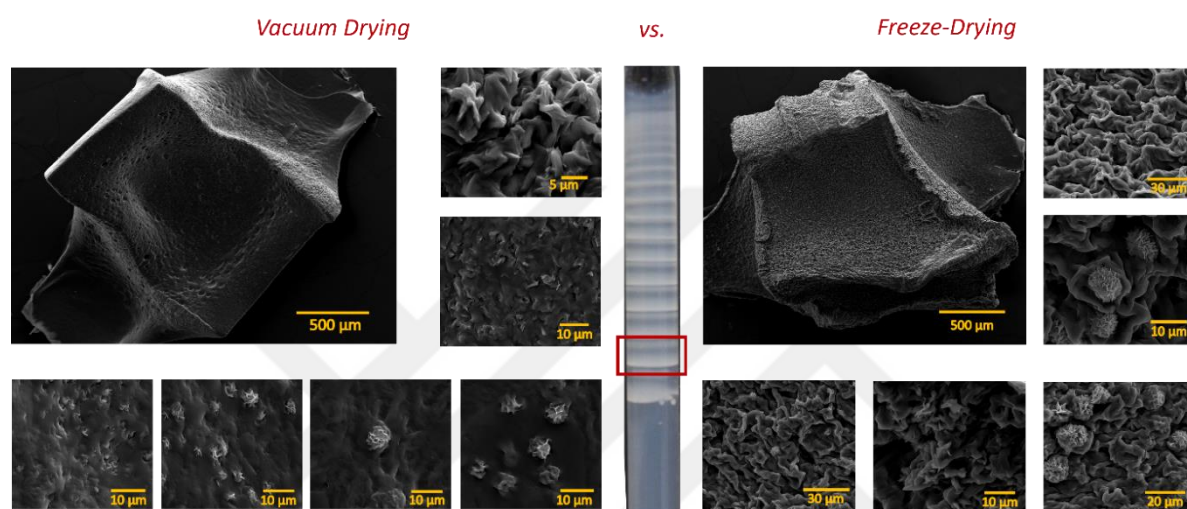


Figure 10. The effect of the drying method on the samples' morphology. The vacuum drying method preserves distinct regions of samples, whereas the freeze-drying method homogenizes gel medium and makes it impossible to differentiate separate regions. Therefore, the former was used in the SEM sample preparation.

SEM imaging and EDX analysis. SEM and EDX were performed with a Quanta 200F model SEM microscope using a 3.5 spot size and 15 kV accelerating voltage. SEM samples were dried using two methods: freeze-drying and vacuum drying. In the freeze-drying method, the samples were frozen using liquid nitrogen and then placed in a vacuum gauge after freezing. In this way, the collapse of the gel structure could be eliminated by sublimation of ice crystals; however, this method led to the homogenization of separate regions of a band (**Figure 10**). Thus, the second method, in which the samples were placed in the vacuum gauge without freezing, was adapted for drying. Even though gel structures collapsed, different areas in a band could be successfully observed.

ATR-IR spectroscopy. A Bruker Alpha Platinum-ATR spectrometer was utilized to obtain IR spectra of samples. After cutting a band in half, the separate regions of the bands were meticulously sliced by contrast difference of the precipitated area. The samples were dried using

the vacuum drying method described above for SEM sample preparation. Resolution and scan number were set to 2 cm^{-1} and 128, respectively, to obtain a high signal-to-noise ratio.

X-Ray Diffractometry. A Malvern PANalytical X'Pert PRO X-ray diffractometer with $\text{CuK}\alpha$ radiation was used for phase characterization. The accelerating voltage and current were 45 kV and 40 mA, respectively. Spire, plateau, void, and spike regions were separated carefully and dried under vacuum before further treatment. The last two forming bands were analyzed between 5 and 65 degrees. Two sets of samples were combined to provide the necessary amount of material to obtain significant peak intensities.

2.8.2 Image Analysis

The LP spacing coefficients were determined using ImageJ and MATLAB software. A line on the picture of the sample, which was taken with a Canon EOS 250D 18-55mm, was drawn in ImageJ. A gray value profile based on red, green, and blue (RGB) colors was plotted using the $\text{gray} = 0.299\text{red} + 0.587\text{green} + 0.114\text{blue}$ formula from this line. The peak points in the graph corresponded to the bands, and the depths represented depleted regions in between bands, as well as void regions within each band. Relatively minor peaks succeeding the higher gray value peaks were those stemming from the next band's spike region (**Figure 11a-b**). While the y-axis represented the gray value profile, the distance from the gel-solution interface was shown on the x-axis. The information provided by this graph was inserted into a home-written

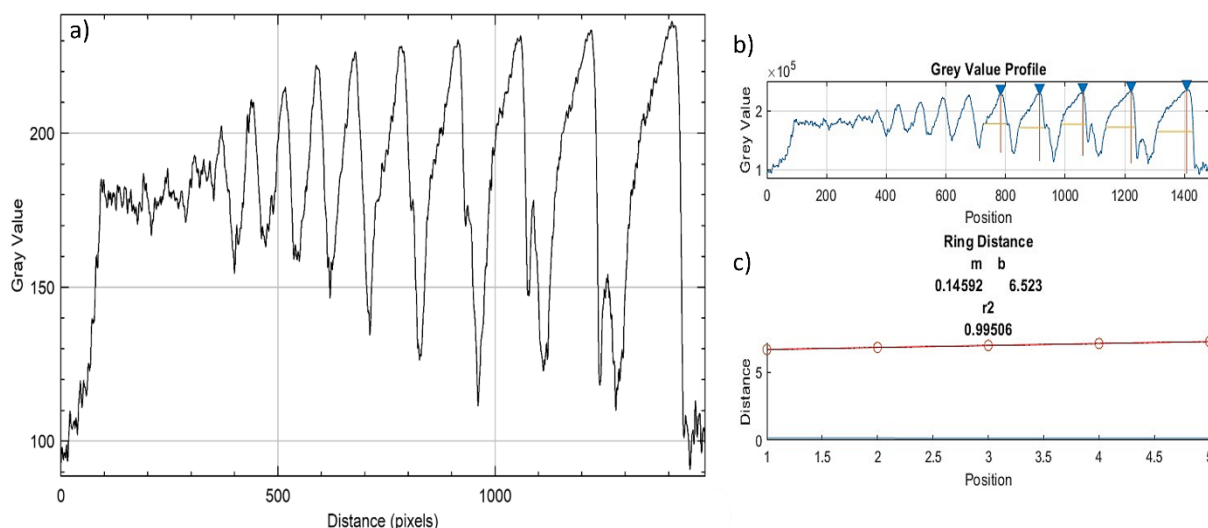


Figure 11. a) The graph of the gray value profile of bands vs. the distance of bands in pixels from the gel-solution interface. b) The same graph created in a home-made MATLAB code with the “find peaks” function. c) The graph of the natural logarithm of band position and band number. The slope gives the spacing coefficient.

MATLAB code to find the peak positions (x_n). The slope of the natural logarithm of the peak position and peak number graph was determined as the spacing coefficient (**Figure 11c**).



CHAPTER 3

3. Results and Discussion

3.1. Magnesium Hydroxide and Calcium Phosphate LP Formation in Blend Hydrogels

In our previous work [60], we formed magnesium hydroxide LPs on agarose and gelatin. The aim was to form LPs of a product that would react with a reactant externally fed to the LPs. The exothermic reaction that takes place on the bands created patterns of infrared radiation – the first example of patterned radiation was shown in the literature. During this work, we noticed that magnesium hydroxide crystal morphologies might be affected by the gel media in which they form. Therefore, the initial studies on the effect of gel blending on the crystal morphology were focused on these LPs. Magnesium hydroxide LPs have the practical advantage that they can be produced in different hydrogels and their blends (**Figure 12**) and are highly reproducible. Nevertheless, like other LP systems, meticulous optimizations of electrolyte concentrations are needed when the gel medium is altered for the neat production of the LPs, and to avoid ‘secondary band’ formation [98], a commonly observed phenomenon for magnesium hydroxide LPs.

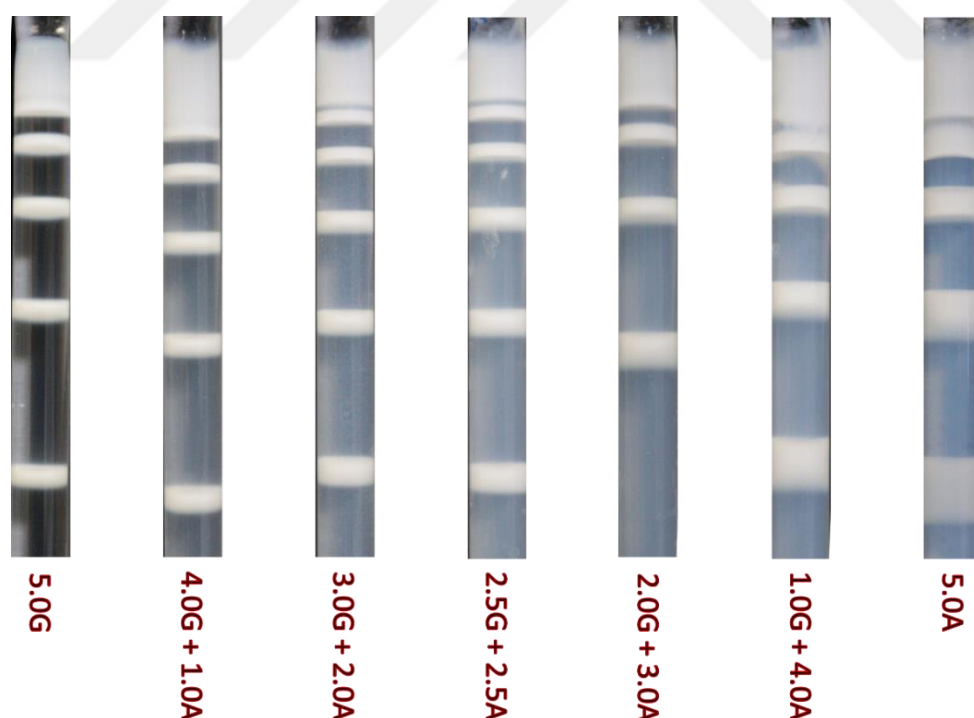


Figure 12. Magnesium hydroxide LPs prepared in gelatin, agarose, and blend hydrogel samples. Spacings and widths of bands changed based on gel composition. G= % (w/v) gelatin and A=% (w/v) agarose.

As shown in (**Figure 13a-g**), for magnesium hydroxide LPs, the blending did not result in a morphology change of magnesium hydroxide crystals formed in the LP precipitates. LPs formed in agarose, gelatin, and their blends exhibited platelet-like morphology across different generations of bands. Later generations of bands displayed bigger particles as reported earlier [61], but the crystal shape remained unchanged for all blend samples. Magnesium hydroxide was also prepared through the wet-chemical precipitation method (**Figure 13h**) to observe whether the gel medium affects the particle morphology. Although the shape of particles was platelet-like, the sizes of platelets were much smaller than those of the gel-mediated process.

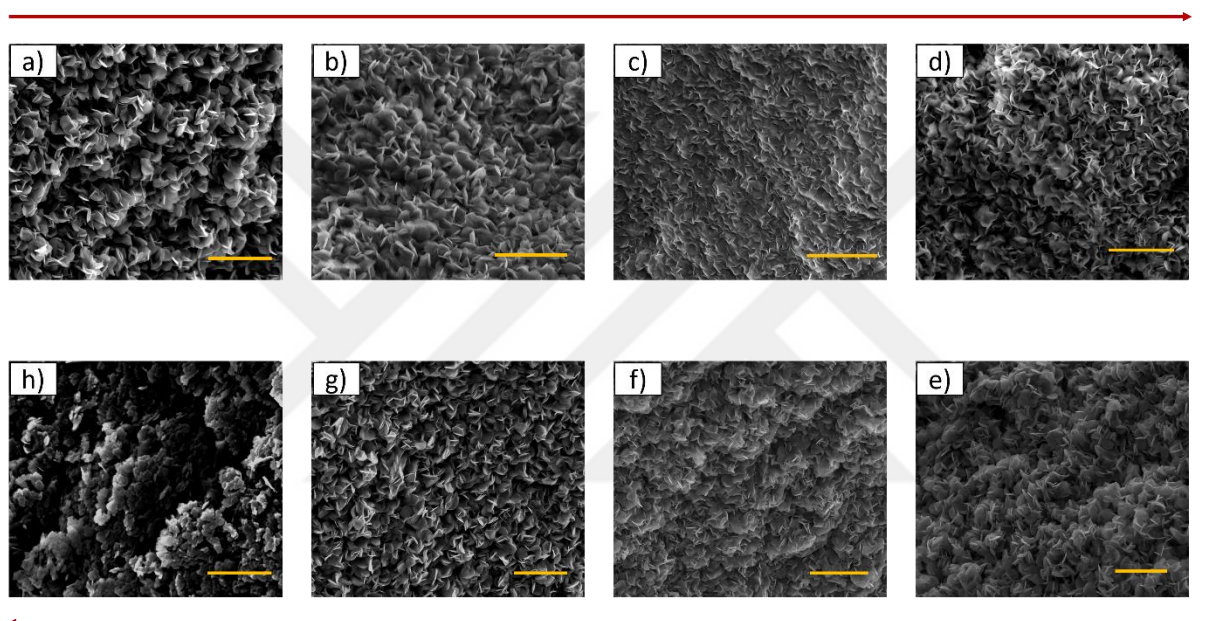


Figure 13. SEM micrographs of magnesium hydroxide LPs in different hydrogels/hydrogel blends shown in **Figure 12**. The ratios of gelatin/agarose are in a) 5.0% to 0, b) 1.0% to 4.0%, c) 2.0% to 3.0%, d) 2.5% to 2.5%, e) 3.0% to 2.0%, f) 4.0% to 1.0%, g) 0 to 5.0%. The second forming band in each sample was imaged for comparison. The samples displayed similar, platelet-like morphologies. The direction of the arrows indicates a decreasing concentration of gelatin. Only in the sample prepared through the wet-chemical precipitation method, shown in (h), is the morphology slightly more irregular than the others. The scale bar is 10 μm for a) to g) and 2 μm for h).

Similar to magnesium hydroxide LPs, CaP LPs are commonly studied in various gel media and can be producible in blend samples as well (**Figure 14a**). Calcium chloride dihydrate was determined to be the inner electrolyte compared to most reported cases (Please see **1.4**), as it deteriorated gelatin medium in control samples when used as the outer electrolyte. In contrast to magnesium hydroxide LPs, CaP LPs form different crystal shapes when prepared in agarose, gelatin, and various blends of these hydrogels. (Further information on the shape changes will be provided in later sections.) The blending not only resulted in the formation of distinctive shapes of particles but also led to the formation of different regions within a single band. When

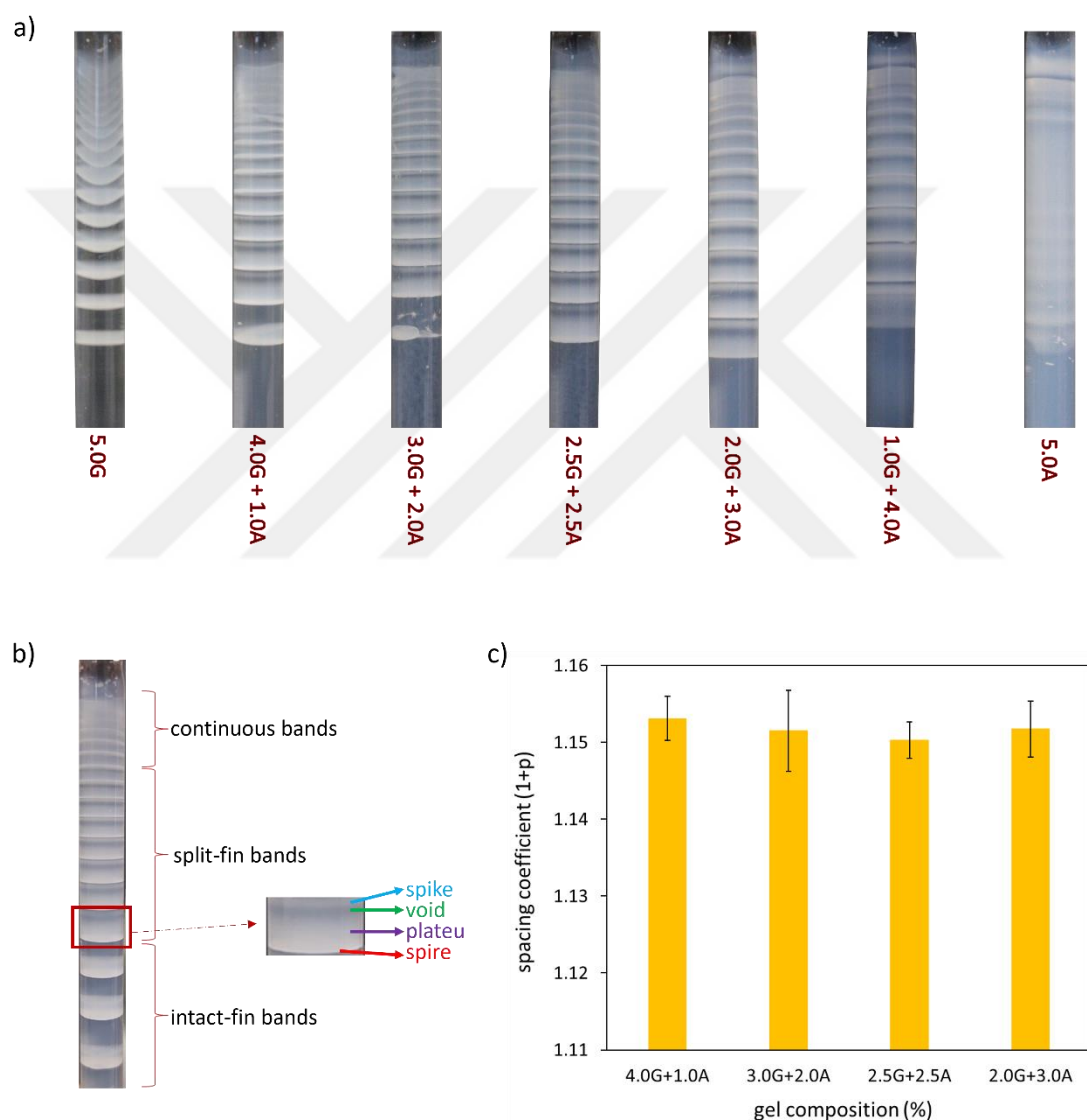


Figure 14. a) CaP LPs prepared in gelatin, agarose, and blend hydrogel samples. Spatial formation and width of bands changed based on gel composition. G= % (w/v) gelatin and A=% (w/v) agarose. b) A single CaP LP in a blend hydrogel. The image shows different types of bands formed in the same sample and different regions of a split-fin band. c) The graph exhibiting the changes in the spacing coefficient based on gel composition.

the LPs formed in agarose and gelatin blend hydrogels, each band consisted of four different regions: spike, void, plateau, and spire. (**Figure 14b**). Jo et al. claimed that this type of band starts to form from the middle (plateau), and then, a void region appears in which the particle density is much lower compared to other regions. When the concentrations of ions increase, again, a spike region appears. The ion concentrations below the plateau increase with time and form the most concentrated part of the band, spire [68]. Due to the complexity of patterns, the spacing coefficient calculations are not easy to perform, and some studies exclude them. In our work, the spacing coefficient of only split-fin bands of the samples was calculated by taking the spire as the reference point. It is crucial to highlight that the analysis is tentative, as bands contain multiple regions, and only one region was used as a reference point for calculation purposes. The results demonstrated that the spacing coefficients of different blends were in similar ranges (**Figure 14c**). The spacing coefficients of samples were determined as 1.1551 (± 0.0029), 1.1535 (± 0.0053), 1.1523 (± 0.0024), and 1.1537 (± 0.0036) for 4.0% (w/v) gelatin + 1.0% (w/v) agarose, 3.0% (w/v) gelatin + 2.0% (w/v) agarose, 2.5% (w/v) gelatin + 2.5% (w/v) agarose, and 2.0% (w/v) gelatin + 3.0% (w/v) agarose, respectively. As the regions in 1.0% (w/v) gelatin + 4.0% (w/v) agarose samples were not well-defined, the spacing coefficient of this specific concentration could not be calculated. Slight changes in other samples can be attributed to the altered gel microenvironments due to blending and their effect on gel properties such as viscosity or porosity.

The optimization of inner and outer electrolytes was made carefully so that patterns could be formed in all gel compositions studied. Overall, the experiments showed that the most convenient system for the regular LP formation was the one with 0.50 M of sodium hydrogen phosphate and 0.020 M of calcium chloride dihydrate, as all the bands were formed fully after 4 days of pattern formation. The blend ratios and the overall concentrations were also optimized to produce regular LPs. Although there were minor differences in the last bands, almost all concentrations produced LP bands successfully in the set of parameters reported in **Figures 14 and 15**. It was observed that the spatiotemporal pattern formation of calcium phosphates is robust against concentration changes of the constituting ions, also in blend hydrogel systems. The robustness of these systems is an important factor in mimicking nature, as ion concentrations or organic content in the natural biomineralization process are prone to dynamic alterations. Therefore, it is reasonable to assume that the blends we formed in this work, consisting of a protein and a polysaccharide, can provide a suitable mimicry of natural media since they can resist alterations in ion concentrations.

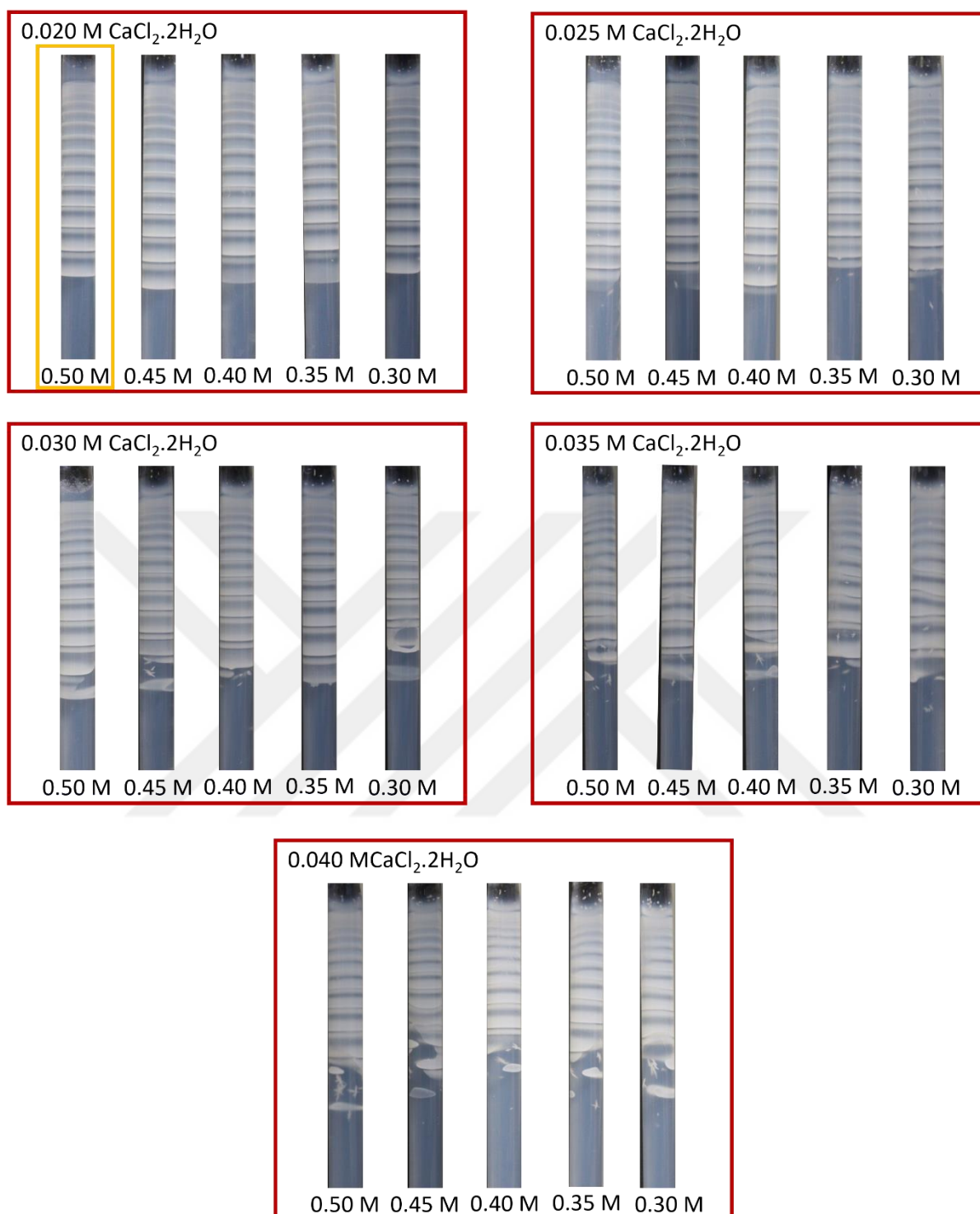


Figure 15. LPs forming in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium with different ion concentrations, in which $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was the inner electrolyte, and Na_2HPO_4 was the outer electrolyte in varying concentrations. The concentrations at the top of each box belong to the inner electrolyte concentration, and the ones given under each sample are the concentration of the outer electrolyte solution.

3.2. The Effect of Synthesis Method on the Phase and Morphology of the CaP Product

3.2.1 CaP Formed via Solution Synthesis

As mentioned above, the synthesis method has a tremendous impact on the morphology of the forming products. Although the local concentration of phosphate ions, thus the local pH of the gel microenvironment, influences the crystal morphology, the hierarchical variety of crystal morphologies cannot be attributed solely to the local ion concentration, and one has to consider the effect of organic matrices and the synthesis method. Therefore, CaP was also prepared via a conventional mixing and precipitation in a solution to observe the morphology of the formed product. The concentration of calcium chloride dihydrate was kept constant while varying concentrations of disodium hydrogen phosphate were used to form precipitate, similar to the formation of precipitates in the LP system, in which the inner electrolyte concentration was constant and the local concentration of outer electrolyte was subjected to changes. Only two main morphologies were observed when using the different Na_2HPO_4 concentrations: small spheres with a size of the order of nm scale, and sheet-like structures. The supersaturation conditions led to the formation of small spheres (**Figure 16a-d**), which is compatible with previous findings in literature (Please see sections 1.1.1 and 1.1.2). Both small spherical and randomly packed morphologies and supersaturation conditions imply that the formed products possibly include ACP. However, Ca/P ratio of the samples was slightly below the minimum reported value for ACP (1.2) in most studies. Still, Qi et al. reported that the minimum ratio can be as low as 1.0 for ACP [99]. Therefore, these results conclude that the main component of the precipitate might be mostly ACP with small amounts of other phases. Lower Na_2HPO_4 concentration conditions, on the other hand, resulted in sheet-like structures, the top parts of which were covered with small and randomly packed spheres. The samples contained both morphology at these concentrations (**Figure 16e-h**). Sheet-like structures are characteristic of DCPD crystals. Yet, Ca/P ratio for these samples was above the reported values. Thus, the product might have contained a combination of various phases. In addition to what has been reported, EDX analysis might not have given a precise Ca/P ratio as the crystals were embedded in a gel medium.

The IR spectra of the products formed at both high (**Figure 16i**) and low (**Figure 16j**) $[\text{Na}_2\text{HPO}_4]$ conditions were also investigated to characterize their structures. At high concentrations, the peaks at around $\sim 1650\text{ cm}^{-1}$ correspond to the -OH group of water molecules [100], whereas the single peaks $\sim 548\text{ cm}^{-1}$ stem from P-O bending vibrations [100]. The absence of a splitting in the peak at 548 cm^{-1} strongly supports the amorphous nature of the

sample, as crystalline structures have a splitting due to the anisotropic environment of phosphate groups [100]. The peak at $\sim 906\text{ cm}^{-1}$ might be the P-O stretching band [100]. Peak splitting was observed at 1033 cm^{-1} and 1083 cm^{-1} , which possibly corresponded to the P-O stretching mode [100], at higher phosphate concentrations. This might be attributed to the

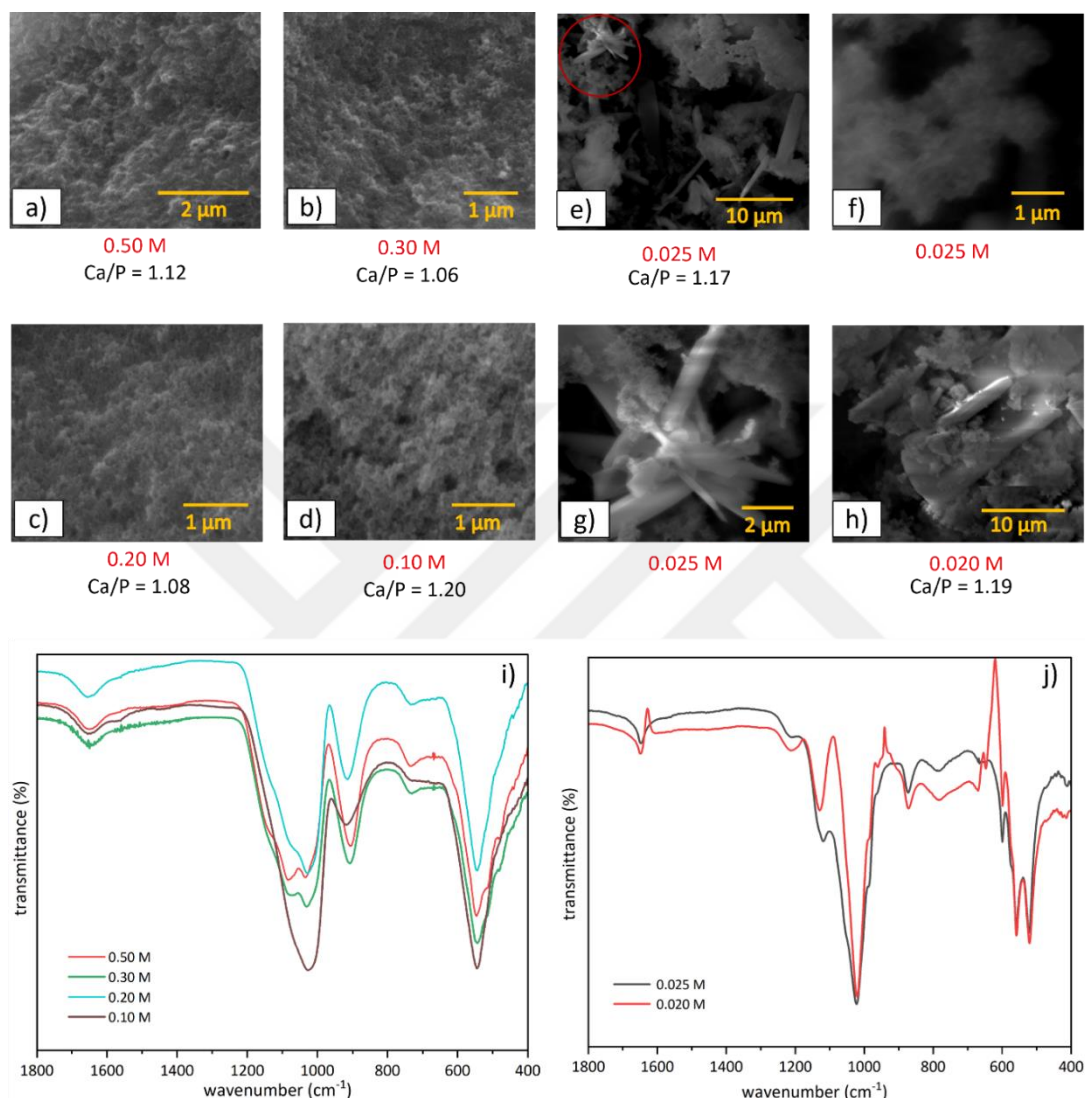


Figure 16. CaP samples prepared through the wet-chemical precipitation method under a-d) high Na_2HPO_4 , and e-h) low Na_2HPO_4 concentration conditions. $[\text{CaCl}_2 \cdot 2\text{H}_2\text{O}] = 0.020\text{ M}$, $[\text{Na}_2\text{HPO}_4]$ is given below the micrographs (in red). Ca/P ratios determined by EDX are given below the images. f) and g) are zoomed areas highlighted with a red circle in e). i) and j) show the IR spectra of the samples.

presence of multiple phosphate environments. Also, the peak shoulders occurring at $\sim 1139\text{ cm}^{-1}$ can be the characteristic P-O stretching mode of ACP structures [100]. Gadaleta et al. suggested that peaks at 1038 cm^{-1} and 1112 cm^{-1} resulted from non-stoichiometric early-stage maturations of HAp molecules or the presence of acidic-phosphate [101]. Also, the broadening

of IR peaks is a clear indication of the lack of crystallinity of samples at higher concentration conditions. As a result, it can be concluded that ACP was the dominant phase at high $[\text{Na}_2\text{HPO}_4]$ conditions, according to the spectra of varying phosphate concentrations, which is in alignment with the Ca/P ratios observed by EDX.

Lower $[\text{Na}_2\text{HPO}_4]$ conditions, on the other hand, resulted in a different crystal structure. The IR peaks of the samples align well with the structure of DCPD. The peaks at $\sim 1647\text{ cm}^{-1}$ correspond to the -OH group bending mode [102]. $\sim 1208\text{ cm}^{-1}$ band attributed to O-H in-plane bending of hydrogen phosphate [102]. P-O stretching mode was observed at $\sim 1119\text{ cm}^{-1}$ [102]. Petrov et al. reported that the band at 988 cm^{-1} and its shoulder at 1005 cm^{-1} stemmed from the symmetric stretching modes of P-O [102]. Our results exhibited a peak at $\sim 1022\text{ cm}^{-1}$ and a small shoulder at $\sim 988\text{ cm}^{-1}$, which can be attributed to the same modes reported. P-O(H) stretching mode appeared at $\sim 872\text{ cm}^{-1}$, which verified the formation of brushite (DCPD) [33]. The peak at $\sim 782\text{ cm}^{-1}$ is associated with the rocking mode of water [102]. Baskar et al. reported that peaks at 527 cm^{-1} and 572 cm^{-1} were the peaks originating from (HO)P=O of acidic phosphates [94]. The peaks at $\sim 522\text{ cm}^{-1}$ and $\sim 559\text{ cm}^{-1}$ might stem from these acidic phosphate species. Another small peak was observed at $\sim 600\text{ cm}^{-1}$, which might be attributed to the presence of other phases. Overall, only two different crystal morphologies and structures were obtained at the given $[\text{Na}_2\text{HPO}_4]$ conditions with the wet-chemical precipitation method.

3.2.2 CaP LPs Formed in Agarose, Gelatin, and Agarose-Gelatin Blends via Reaction-Diffusion

As pictured in **Figure 8**, CaP forms LP bands in agarose and gelatin. In agarose, the formation is less prominent, in gelatin, distinct and regular bands form. The CaP produced in gelatin medium exhibited agglomerated-crumpled (**Figure 17a**) or flake-like morphologies (**Figure 17b**). On the other hand, in earlier precipitate formation, dendritic morphology was dominant in agarose (**Figure 17c**), whereas the bands and continuous precipitate regions of calcium phosphates displayed a morphology that is generally referred to as “nanoflower” (**Figure 17d-f**). In such a morphology, the particles are agglomerated in a way that a spherical morphology with a highly porous structure is obtained. It was also determined that the inside of nanoflower structures consisted of agglomerated particles of CaP (**Figure 17e, inset**).

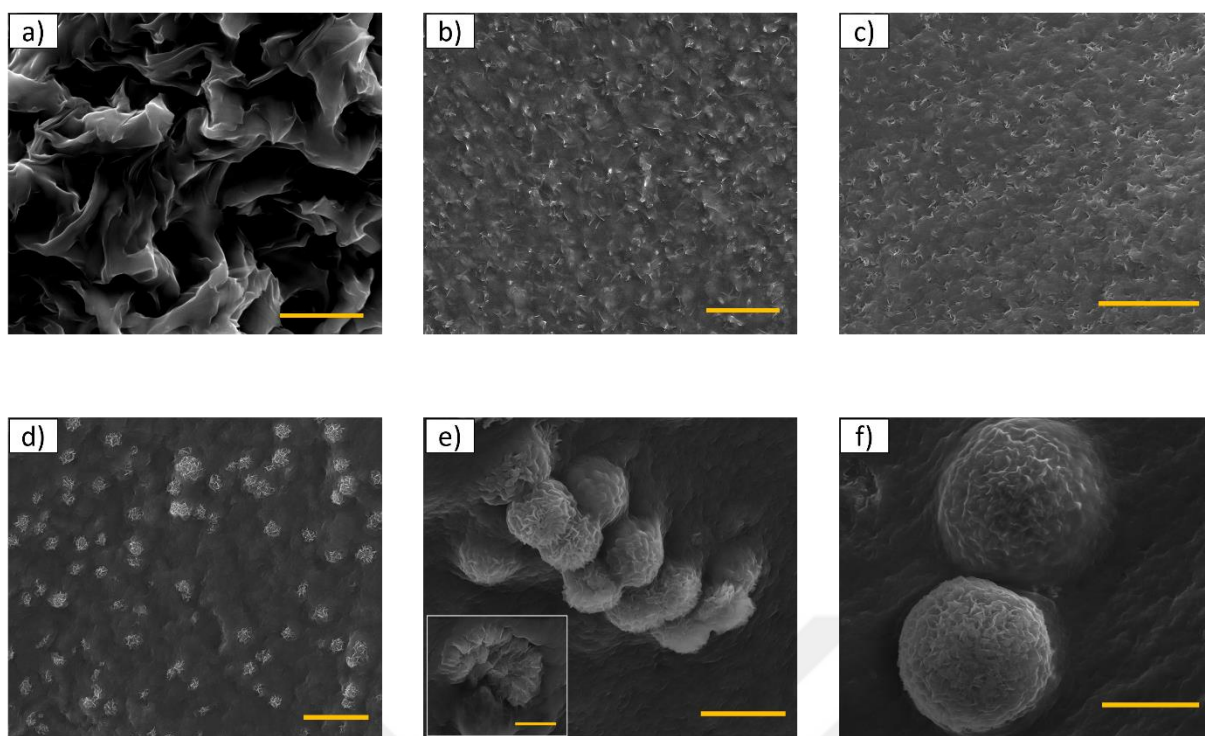


Figure 17. The micrographs of CaP formed in LPs in agarose or gelatin. a) and b) belong to the spire and plateau regions of 5.0% (w/v) gelatin samples, respectively. c) and d) are the micrographs of the last band above continuous precipitation in 5.0% (w/v) agarose sample. e) is the product in the continuous precipitation region observed in the middle part of 5.0% (w/v) agarose sample. Inset: the inside of spherical structures. f) is the first band forming after continuous precipitation. The scale bar is 10 μm for b), c), d), e), and f), 5 μm for a), and 4 μm for the inset figure of e).

As depicted in **Figure 14b**, when the LPs formed in agarose and gelatin blend hydrogels, each band consisted of four different regions: spike, void, plateau, and spire. In the literature, inhomogeneous crystal densities within a band in LPs are reported [88][89]. However, there is only one study showing 4 distinct regions within a band [68]. Therefore, we focused our analysis on the within-band variety of particles across different generations of bands. As the best macroscopic reproducibility and microscopic differences within the bands and the band regions were obtained in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium (**Figure 14a** and **Figure 19**), the rest of the analysis of the regional products was performed with these samples.

Before setting a hypothesis on the gel-induced formation of CaP products and the effect of blending on the formed product and its morphology, it should be confirmed that the distributions of the polymers in the blends are spatially homogeneous. Therefore, 3 different parts (bottom, middle, and top) of blends gelled in the Pasteur pipette were investigated to

understand whether the samples were homogeneous across different locations. The IR spectra of blank blend samples (Please refer to **2.1**) prepared showed that the signals observed in the blends are mathematical sums of individual signals of agarose or gelatin (**Figure 18a-c**). The different parts showed similar spectra, proving reproducible minimal heterogeneity. The IR spectra taken after the addition of the inner electrolyte to the blend systems were also similar to those of the blank blend samples (**Figure 18d-f**).

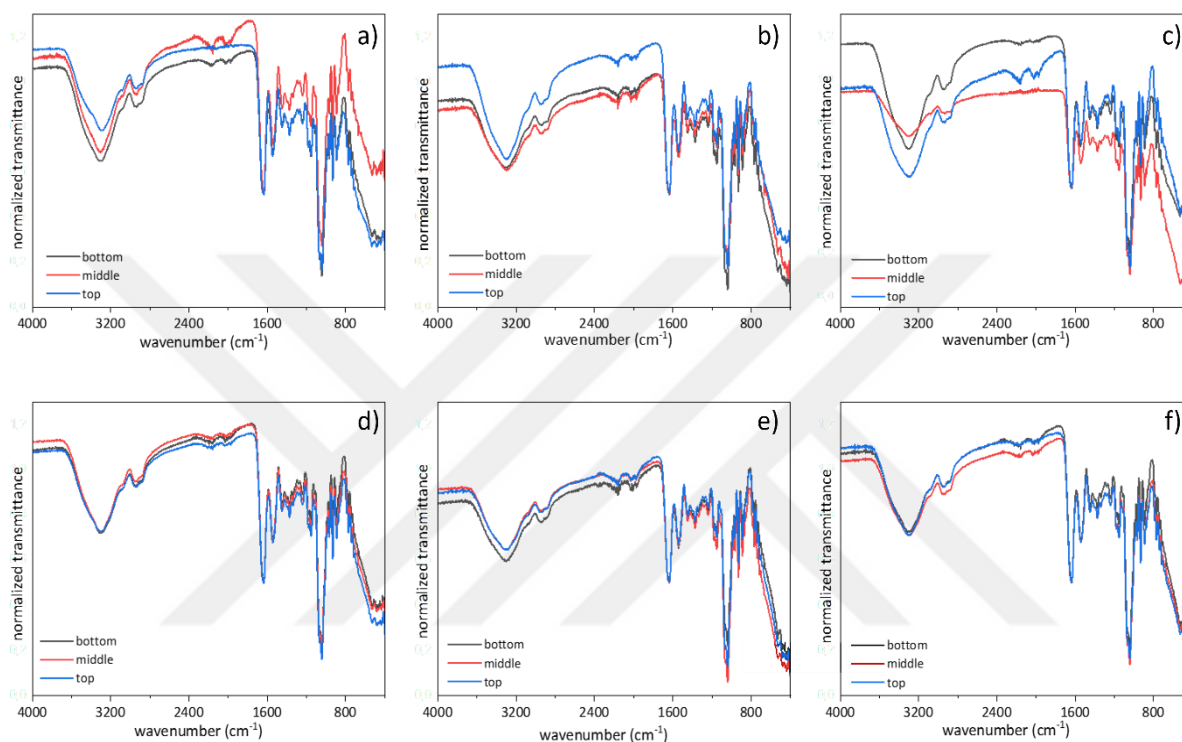


Figure 18. The IR spectra of a-c) 3.0% (w/v) agarose + 2.0% (w/v) gelatin blank gel samples, d-f) 3.0% (w/v) agarose + 2.0% (w/v) gelatin samples with 0.020 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ across different parts in the gel (named as bottom, middle, and top).

Consequently, the CaP products that formed in the blend samples were imaged by SEM. The SEM micrographs (**Figure 19**) exhibited different morphologies of particles across distinct regions of each band. Interestingly, in varying agarose/gelatin ratio blends, the same regions displayed very similar CaP particle shapes. This finding supports the hypothesis that the shapes of particles are invariant against the organic composition of the media in which particles form. On the other hand, as the agarose content increased and the gelatin content reduced, fewer and slightly bigger crystals developed. This result can be attributed to the lower amount of gelatin, providing fewer nucleation sites.

Morphology of the CaP formed in the different regions of the bands.

- **In the spire regions** of the different-ratio blends, agglomerated-crumpled morphology was dominant in all samples (**Figure 19**), similar to the case of gelatin control samples (**Figure 17a**).
- **The plateau regions** exhibited two kinds of particle shapes: flake-like shapes, forming in most of the plateau region (with higher density near the spire), and star-like or dendritic shapes near the void region (**Figure 19**). Star-like morphology was observed near the void region as the crystal density and the number of nuclei decreased, promoting anisotropic crystal growth. Close to the spire region, however, the nucleation was high enough for crystals to adopt flake-like shapes, which prevented the development of nanoflower or dendritic structures.
- **In the void region**, a limited number of crystals and nucleation sites resulted in the formation of nanoflower structures in which crystals isotropically and hierarchically grew in multiple dimensions.
- **In the spike region**, particles with two distinct crystal shapes formed: flake-like particles in the earlier formation of the spike and star-shaped crystals near the void. The differences between the crystal number and size in the spike and plateau region were a direct result of the band formation mechanism.

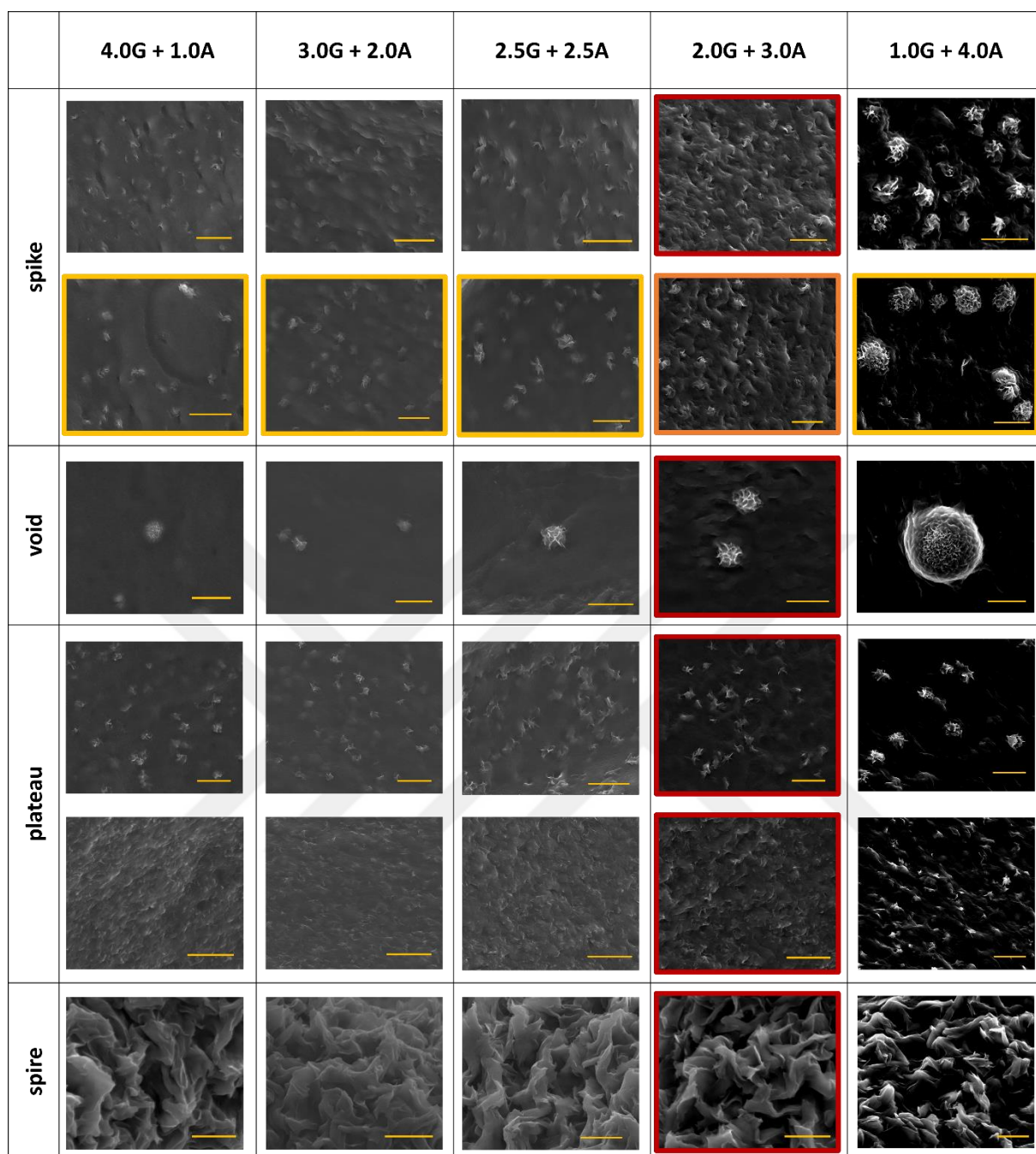


Figure 19. The micrographs of crystals forming in different regions of an LP band and varying gel concentrations show the various morphologies of CaP obtained within a single band in the LP. G and A represent % (w/v) gelatin and % (w/v) agarose, respectively. The red framing shows different regions of 3.0% (w/v) agarose + 2.0% (w/v) sample, while the yellow framing demonstrates how the particle size and number change as a function of blend composition. The scale bar is 5 μm for spire regions and 10 μm for plateau, void, and spike regions.

The regional morphology trends in the spire-plateau-void-spike regions in the 3.0% (w/v) agarose and 2.0% (w/v) blends repeated themselves in each new generation of the LP bands. This trend was slightly lost when the agarose/gelatin ratio increased to 4.0% (w/v) agarose and 1.0% (w/v) gelatin. In these blends with higher amounts of agarose, different generations of bands contained particles with various morphologies in different regions of the bands (**Figure 20**). In summary, blending resulted in 1) LP bands that were divided into some regions, the

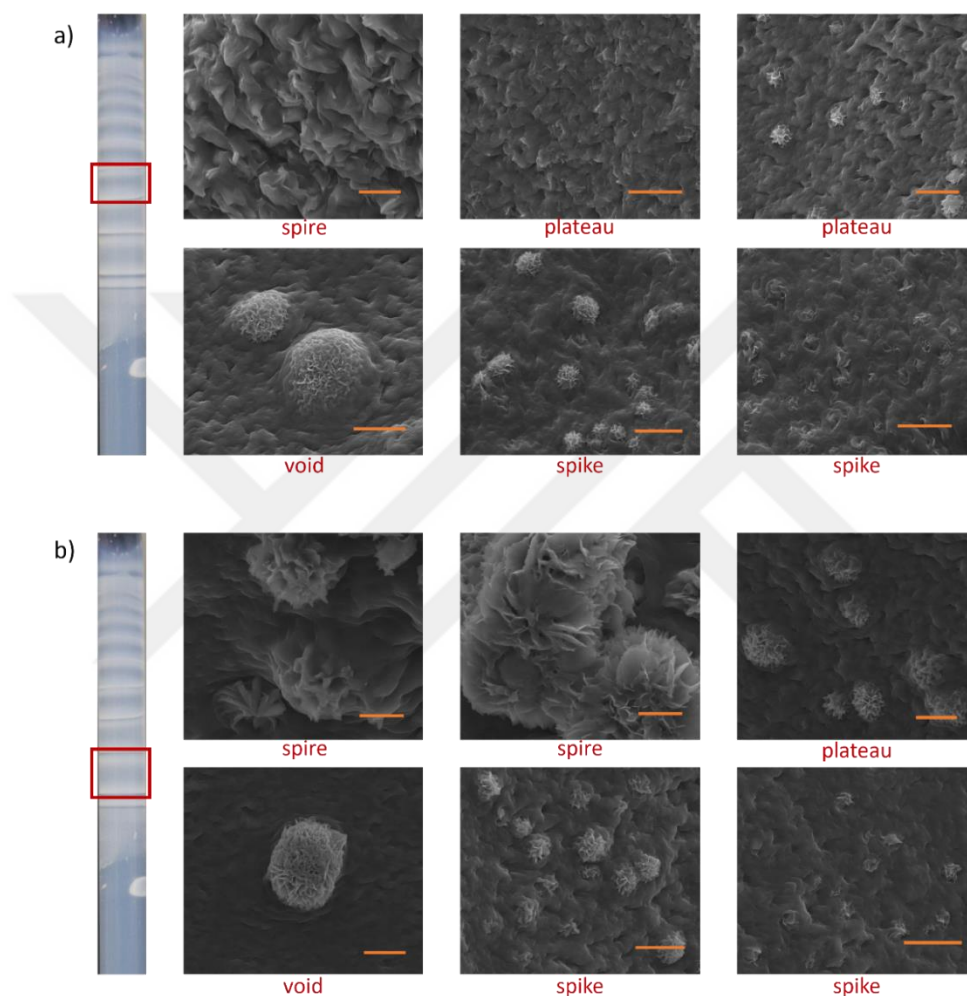


Figure 20. Spatiotemporal formation of LPs in 4.0% (w/v) agarose + 1.0% (w/v) gelatin blend sample. a) and b) exhibit crystal morphologies of distinct regions in different generations of bands. The scale bar is 5 μm for the spire and 10 μm for the other regions.

spire-plateau-void-spike regions, in which the characteristic morphology of the CaP repeated itself in each generation of the LP – such a behaviour cannot be observed in only gelatin or only agarose serving as the LP formation medium. 2) The increase in the amount of agarose created an instability for the repeating morphology across the generations, presumably because of the decrease in the nucleation sites and harder formation of the LPs.

The identity of the CaP formed in the different regions of the bands.

Ca/P ratio comparison is one of the methods to identify the crystal structures of different phases of calcium phosphates. The EDX analyses of the products formed in different regions of the LP bands in the samples showed that the Ca/P ratio did not vary significantly in these regions. There was also no significant change in the Ca/P ratio of the products formed in different agarose/gelatin ratio blends (**Table 4**). The observed Ca/P ratio values were close to that of OCP (Ca/P = 1.33). Therefore, it was reasonable to assume that the samples contained mostly OCP with other phases present in small proportions.

Table 4. Ca/P ratio of different regions of control and blend samples.

	spire	plateau	void	spike
5.0% (w/v) gelatin	1.22 - 1.41	1.20 - 1.46		
4.0% (w/v) gelatin + 1.0% (w/v) agarose	1.19 - 1.27	1.28 - 1.39	1.30 - 1.32	
3.0% (w/v) gelatin + 2.0% (w/v) agarose	1.23 - 1.63	1.29 - 1.39	1.15 - 1.41	1.03 - 1.42
2.5% (w/v) gelatin + 2.5% (w/v) agarose	1.24 - 1.36	1.18 - 1.66	1.23 - 1.38	1.20 - 1.45
2.0% (w/v) gelatin + 3.0% (w/v) agarose	1.28 - 1.29	1.0 - 1.36	1.15 - 1.42	1.0 - 1.47
1.0% (w/v) gelatin + 4.0% (w/v) agarose	1.24 - 1.25	1.20 - 1.34	1.18 - 1.26	1.06 - 1.37
5.0% (w/v) agarose	1.14 - 1.34			

The ATR-IR spectra can also provide some information about the formation of the CaP product. Particularly, some of the characteristic peaks, e.g., PO_4^{3-} or OH^- peaks in the structure of HAp, can be determinative in identifying the particular crystal structure. However, our products were formed as minute amounts of material in a gel medium; therefore, most of the IR peaks stemming from CaP precipitates were suppressed by the peaks arising from the gel. Nevertheless, three newly arising peaks/shoulders appeared in the spectra of the LP-containing samples that could be attributed to the formation of CaP (**Figure 21**). **Figure 21** demonstrates representative examples of these spectra. The CaP signals in the spectra are the O-P-O bending detected at $\sim 550\text{-}560\text{ cm}^{-1}$ and $\sim 590\text{-}605\text{ cm}^{-1}$, and the shoulder at $\sim 1010\text{-}1025\text{ cm}^{-1}$ originated from the P-O asymmetric stretching mode [53]. However, several phases of CaP have been reported to contain the IR bands at the same regions, and there were no characteristic IR bands

for any CaP phase. Thus, IR analysis was not conclusive for structure identification, even though it confirmed that each sample contained the CaP IR bands at similar wavenumbers.

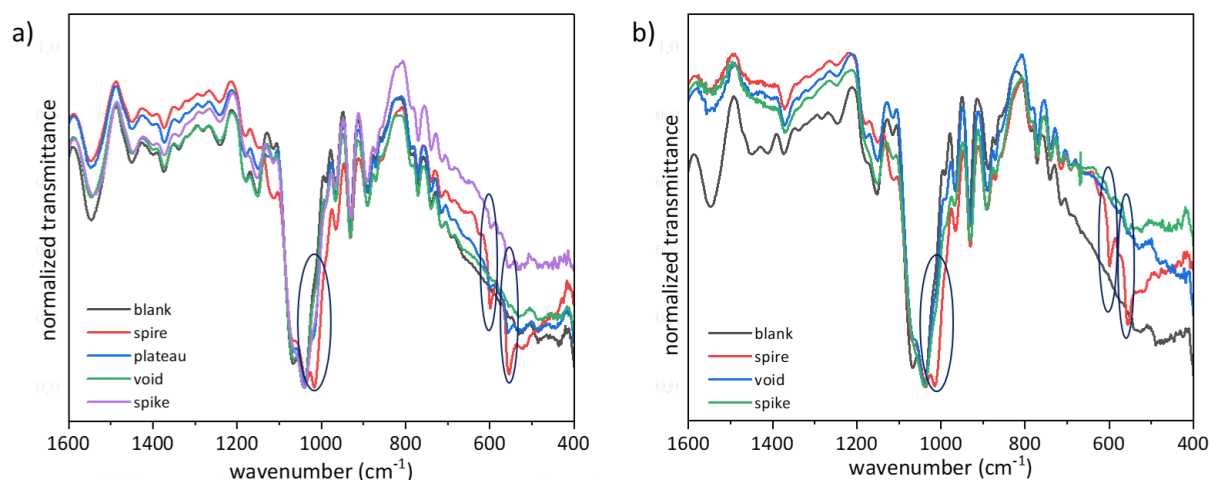


Figure 21. The IR spectra of a) 3.0% (w/v) agarose + 2.0% (w/v) gelatin and b) 4.0% (w/v) agarose + 1.0% (w/v) gelatin. The new peaks/shoulders (circled on the spectra) arise from the CaP product.

The XRD diffractogram can provide conclusive identification of the different phases of CaP. As the sample size was minute (<1 mg) in its powdered form, the XRD analysis of CaP samples was performed in their gel-embedded form. In this case, the signal-to-noise ratio of XRD signals remained low, and the signals appeared broader. Also, the small sample size within the gel medium led the peaks to be less intense compared to the minerals in their free, powdered forms. Nevertheless, we analyzed the XRD diffractograms of the different regions in a band of an LP-forming blend sample (**Figure 22**). The spire region of the second band was found to display the peaks most clearly among the diffractograms of all regions. The major XRD peaks of the spire region of the second band were observed at 27.15° , 28.50° , 31.73° , 33.50° , 42.90° , and 52.91° . Relatively less intense patterns were obtained at 27.82° , 35.13° , 36.62° , 38.05° , 40.87° , 46.34° , 50.87° , 59.08° , and 62.00° (**Figure 22a**). The reference patterns of possible products are also given in **Figure 22a**, and it has been confirmed that all the peaks are well-aligned with OCP, whereas no peaks are found to originate from other phases (e.g., HAp (25.88°) or CDHAp (25.85°)). This, combined with Ca/P ratio of the samples obtained from EDX analysis, strongly suggests that the crystal structure of the formed products consists mostly of OCP and (maybe) smaller amounts of other phases. Yet, the other phases' amounts are quite low that their signals do not appear in the diffractograms. On the other hand, the XRD patterns of the powdered CaP sample synthesized through the wet-chemical precipitation method were completely aligned with the DCPD structure, and none of the peaks were

attributable to other phases. Therefore, it was concluded that the gel-mediated process yields distinct structures from those samples produced with different synthesis methods.

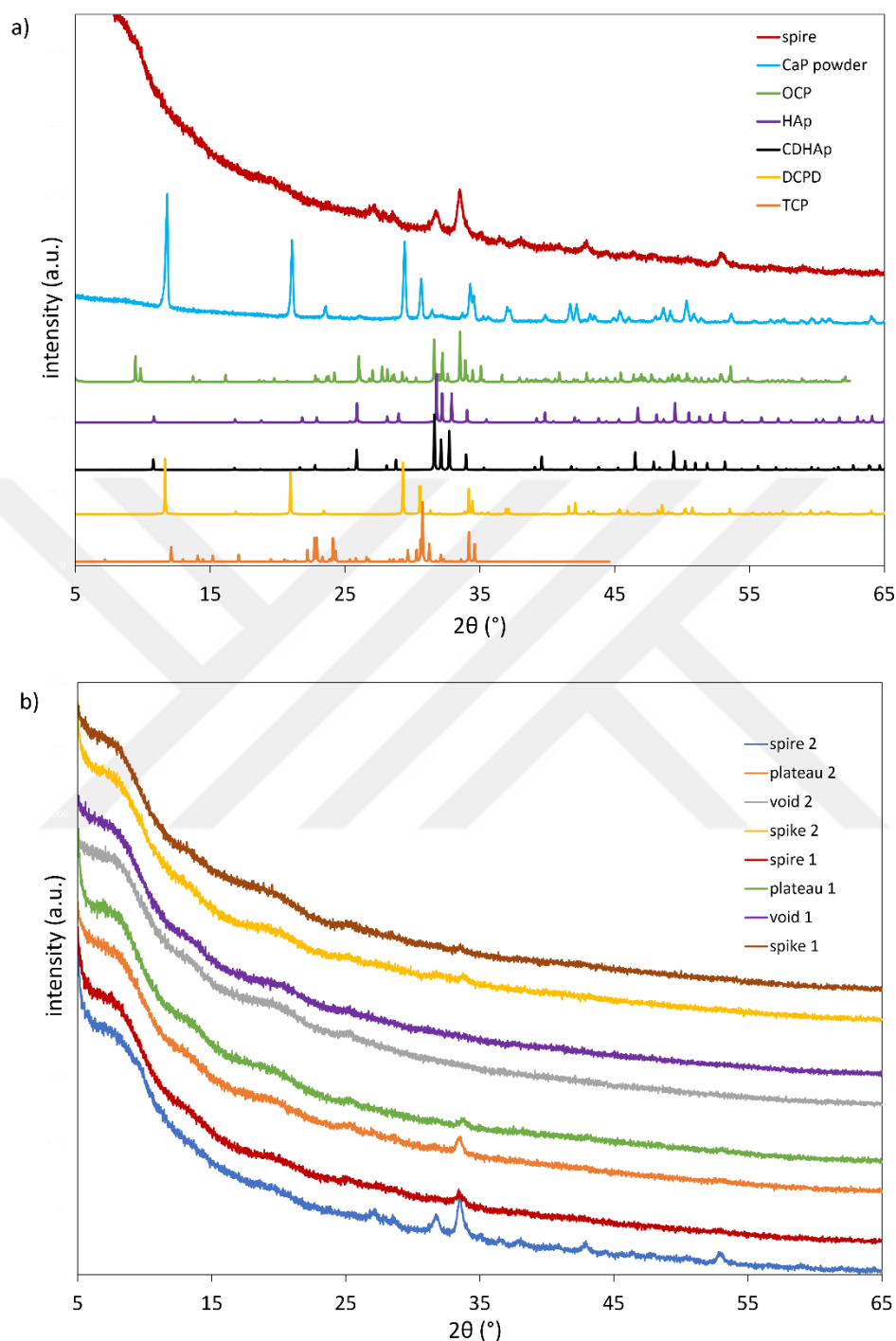


Figure 22. XRD diffractogram of samples formed in 3.0% (w/v) agarose + 2.0% (w/v) gelatin. Comparison of a) the LP sample and powdered sample diffractograms with possible phases present in the samples and b) distinct regions of different bands. 1 refers to the last forming band, while 2 corresponds to the second-to-last band. Reference patterns are OCP: 00-074-1083 (ICDD), HAp: 00-009-0432 (ICDD), CDHAp: 01-073-2657 (ICSD), DCPD: 01-072-1240 (ICSD), and TCP: 00-009-0348 (ICDD).

3.3 Experiments for CaP Formation under Different Experimental Conditions

3.3.1 The Effect of Gel and Outer Electrolyte Concentration on Crystal Morphology

In most studies, it has been reported that the crystal morphologies of calcium phosphates [49][103] and Liesegang patterns [81] are highly dependent on the concentrations of gel and/or the electrolytes, as they directly govern the diffusion rate of ions or the level of supersaturation. Therefore, it is crucial to perform experiments with varying gel and electrolyte concentrations to observe how robust our blend systems are against alterations of the chemical parameters. In this part of the work, individual gel concentrations and the outer electrolyte concentration were variables, while the concentration of the inner electrolyte was constant. The diagram below displays how crystals were shaped in each region of a band, prepared under different phosphate ion and gel concentration conditions.

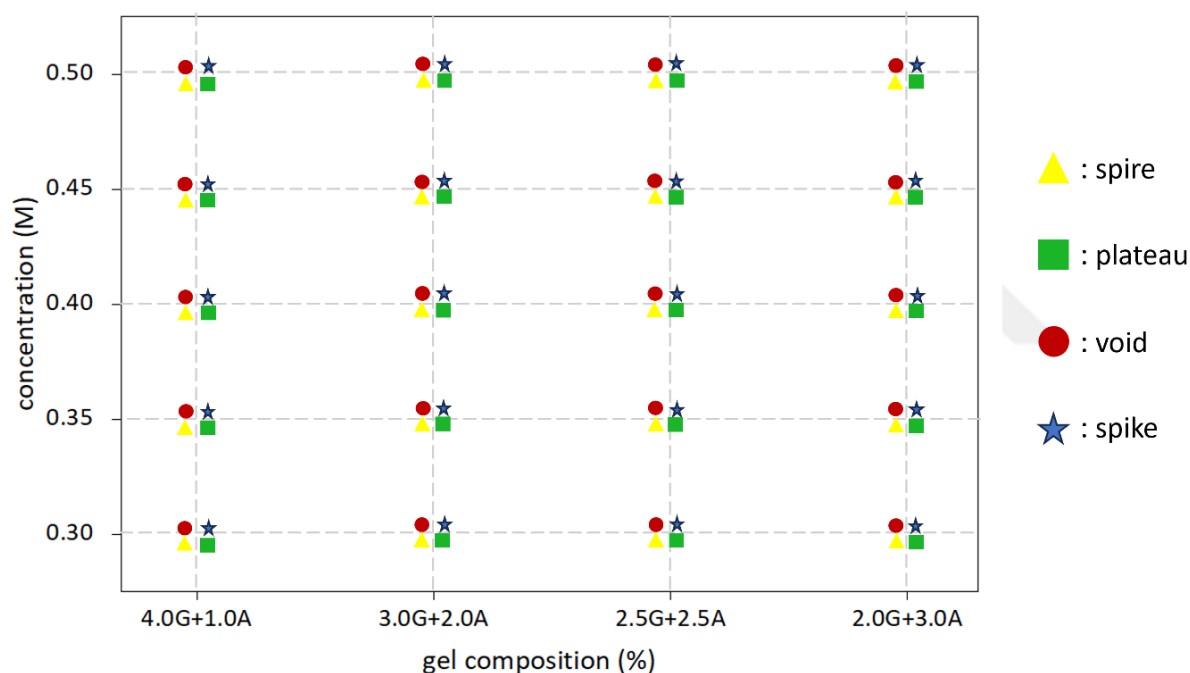


Figure 23. The morphology diagram displaying how gel and outer electrolyte concentrations affect the particle morphology in different regions of a band. The morphologies of crystals are color-coded. Yellow, green, red, and blue represent agglomerated-crumpled morphology, flake-like and star-like morphology, nanoflower structures, and flake-like morphology in lower density with star-like morphology, respectively.

The morphologies obtained at the spire-plateau-void-spike regions repeated independently of the outer electrolyte concentration and gel composition. The numbers and sizes of particles changed under varying concentration conditions, but the shape remained almost the same in the same zones of different samples. (Except the case of 4.0% (w/v) agarose + 1.0% (w/v) gelatin blend samples, where the morphological instabilities start to emerge as

discussed above). For example, the product in the void region almost always exhibited nanoflower morphology, while agglomerated-crumpled morphology was the only type of morphology in the spire regions across the different systems (**Figure 23**). That is, both macroscopic and microscopic levels of patterning remained to form under different concentration conditions in a hierarchical manner. These findings strongly suggest that blend gel systems of calcium phosphate LPs are robust in terms of the formed product morphology within a range of ion and gel concentrations, contrary to what has been reported.

3.3.2 Experiments for CaP LP Formation at Varying Temperatures

As previously discussed, both LP formation and biomineralization processes are influenced by several experimental parameters. That is why the question of “How do the crystal morphologies of biominerals form and adapt under varying conditions?” should be answered to understand the underlying mechanisms of pattern formation and biomineralization. Temperature has a drastic effect on both processes as it directly governs the diffusion rate of ions and the kinetics of the reaction or reaction rate. Bruggeman equations show how the diffusion of ions takes place in a porous medium [104]. In such a model, effective diffusion is directly correlated with the Stokes-Einstein equation (*Equation 2*). According to the equation, the diffusion coefficient (D) is directly proportional to the temperature (T), in which k , η , and a are the Boltzmann constant, dynamic viscosity, and Stokes radius, respectively [105]. Thus, diffusion of ions in a porous medium is expected to be limited at lower temperatures. Similarly, the reaction rate (k) is also assumed to be declining with decreasing temperatures (T) according to the Arrhenius equation (*Equation 3*), where A , E_a , and R are the frequency factor, activation energy, and gas constant, respectively [105].

$$D = \frac{kT}{6\pi\eta a} \quad (\text{Equation 2})$$

$$k = Ae^{-E_a/RT} \quad (\text{Equation 3})$$

Therefore, CaP LPs forming at different temperatures may have different macroscopic and microscopic properties. It has been reported that the solubility of different calcium phosphate phases decreases with increasing temperature [47], which implies that we can expect significant differences in the positions of the LP bands. In this work, we formed CaP LPs at room temperature (25 °C), 4.0 °C, and 37 °C (to mimic the physiological temperature at which the natural biomineralization process takes place) (**Figure 24**) to investigate these differences.

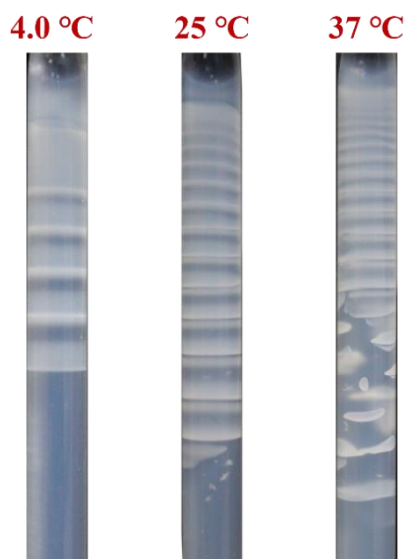


Figure 24. CaP LPs prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium at different temperatures.

As expected from a precipitate that has a lower K_{sp} at higher temperatures, CaP forms fewer bands with a higher p-value at lower temperatures. CaP LPs prepared at lower temperatures (4.0 °C) also had morphologies that were more uniform (small, platelet-like particles) across different regions of the bands. This might be because both diffusion and kinetics are limited drastically, minimizing the local differences stemming from blending. In addition, the higher solubility of the precipitate may have contributed to the observation of the same morphology throughout the LP (**Figure 25**, purple-framed regions). When the temperature was raised to the room temperature (25 °C), diffusion and reaction rates increased, while the solubility decreased; each region within the bands exhibited its unique morphologies (**Figure 25**). The last set of LPs formed at 37 °C demonstrated that within-band variety was sustained at higher temperatures, even though more and larger particles were observed in most parts of the band (**Figure 25**, red-framed regions). Considering the Stokes-Einstein and Arrhenius equations, both the diffusion coefficient and the reaction rate increase at elevated temperatures. Enhancing ion mobility and reaction rate might lead to higher local supersaturation levels in shorter times, resulting in more nucleation and faster growth of particles. Thus, more and larger particles can be expected when the solubility decreases at higher temperatures.

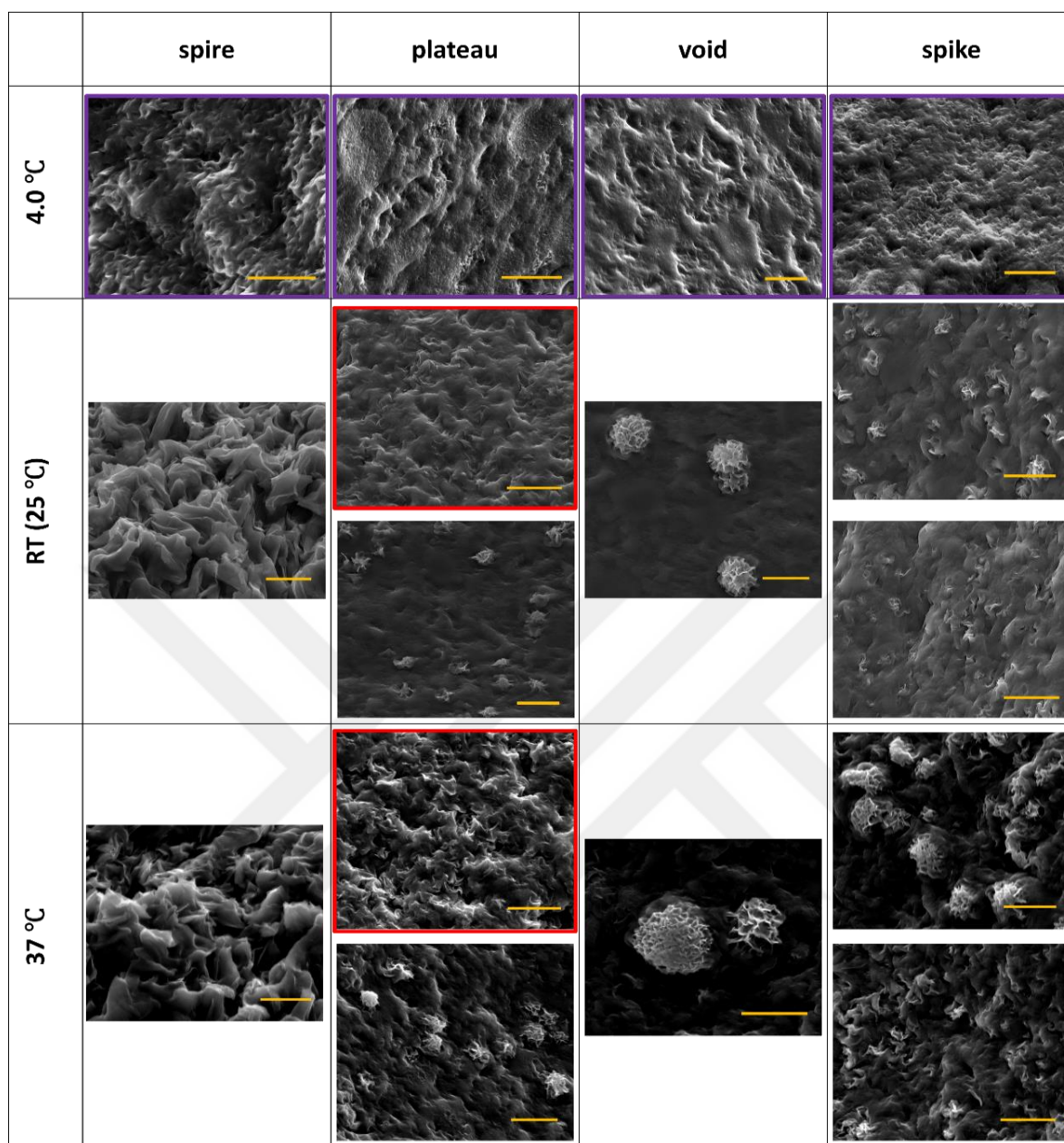


Figure 25. SEM micrographs of crystals formed in different regions of a band (in 3.0% (w/v) agarose + 2.0% (w/v) gelatin) at 4.0 °C, 25 °C, and 37 °C. The purple and red frames are used to show uniformity of particle shapes and an increase in the number and size of particles, respectively. The scale bar is 5 μm for the spire and 10 μm for the other regions.

Table 5 presents Ca/P ratio of different regions of bands prepared at different temperatures. It is expected that the temperature has an impact on the solubility and, thus, the crystal structures of the formed products. However, Ca/P ratios obtained from the EDX analysis of the samples were not significantly different for different regions (e.g., the differences between plateau and spike regions at different temperatures), implying that the temperature

difference did not lead to a significant change in the CaP crystal structures, which might be concluded to be OCP in all cases.

Table 5. Ca/P ratio of different regions of samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium at different temperatures.

	spire	plateau	void	spike
4.0 °C	1.26 - 1.30	1.18 - 1.27	1.22 - 1.59	1.06 - 1.53
25 °C	1.28 - 1.29	1.0 - 1.36	1.15 - 1.42	1.0 - 1.47
37 °C	1.21 - 1.28	1.06 - 1.57	1.14 - 1.30	1.12 - 1.31

3.3.3 Experiments for CaP LP Formation under Varying pH Conditions

One of the most prominent parameters for both LP formation and biomineralization is the effect of local pH conditions at which precipitate forms. In CaP LP samples, the influence of pH can be investigated in two aspects: the pH of hydrogels (especially those with charged functional groups) and the pH of phosphate species that is used to generate bands. Therefore, pH arrangement is crucial to understanding how the formation of macroscopic bands and their microscopic properties would be influenced. In our system, arranging the pH of both gel and phosphate-containing species is critical to analyze the importance of the presence of different functional groups, acting as nucleation centers, and different phosphate species on band formation. **Figure 26** displays how ions might be interacting with the gel environment and particles form in our blend system without any alterations.

As mentioned, agarose is a relatively inert matrix with a neutral structure, making it less susceptible to ion-gel interactions. Hydroxyl groups in the structure of agarose (**Figure 27b**) might interact very weakly with calcium ions; however, the interactions would be much weaker than electrostatic interactions between gelatin and calcium. The structure of gelatin, on the other hand, contains charged functional groups such as carboxylate, stemming from glutamic acid, and guanidinium, originating from arginine (**Figure 27a**). The presence of charged functional groups in gelatin leads to electrostatic interactions between these groups and ions. It can be concluded that positively charged calcium ions might interact with negatively charged carboxylate groups, whereas negatively charged phosphate species might prefer to attach positively charged guanidinium groups. Therefore, the simplified diagram in **Figure 26** can be utilized to demonstrate how the structure of gelatin might act as a nucleation platform.

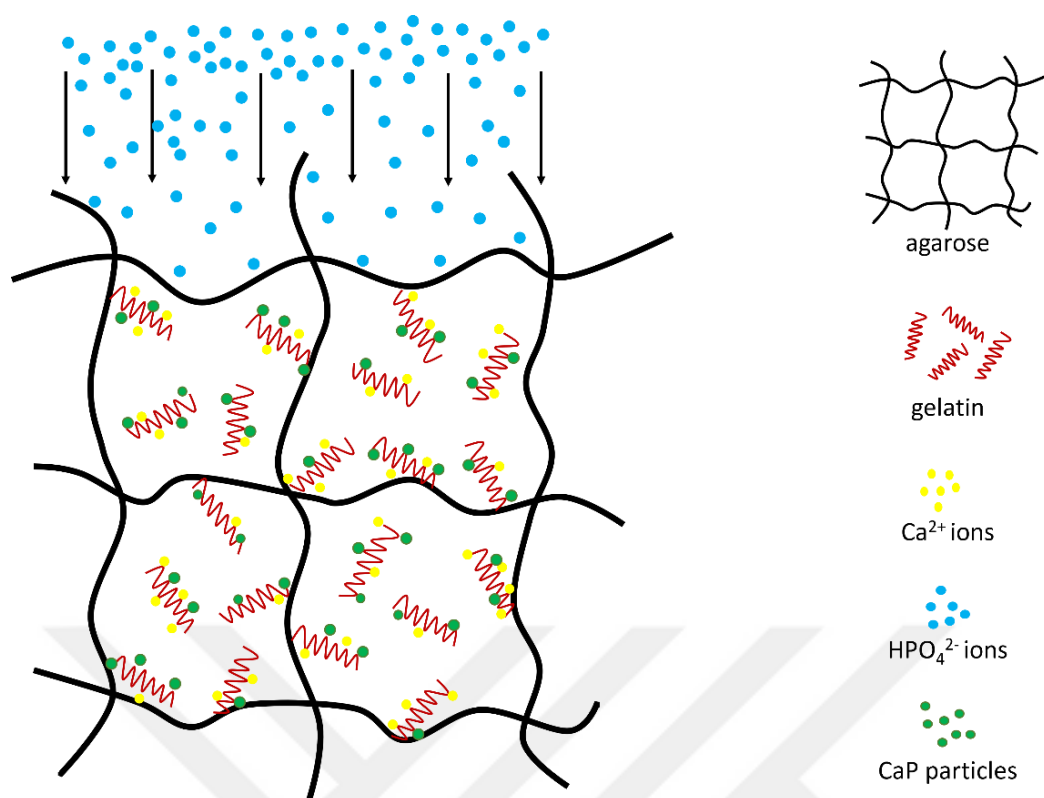


Figure 26. Possible interactions between the ions with the gel structure and the CaP particle formation. The arrows indicate the direction of diffusion.

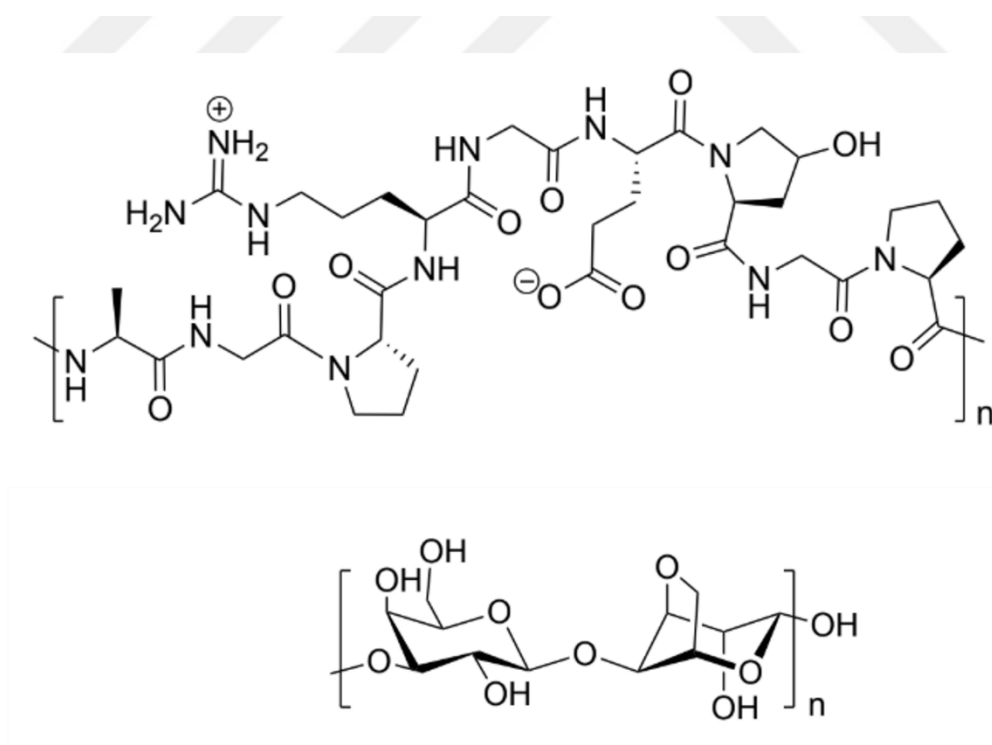
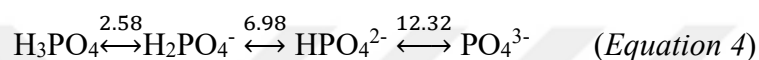


Figure 27. The chemical structures of gelatin (top) and agarose (bottom).

The preparation details for the samples related to this part of the study are given in Section 2.4. Briefly, the LP forming systems were prepared with the gel pH of 4.0, 5.5, and 12, and the outer electrolyte pH of 5.5, 7.0, 9.0, and 10.5. The outer electrolyte pH of 5.5 did not lead to the formation of patterns in each gel pH. Equilibria between phosphate species [106] (*Equation 4*) demonstrate that pH 5.5 corresponds to dihydrogen phosphate as the dominant phosphate species, with small amounts of phosphoric acid and hydrogen phosphate. It is a known fact that dihydrogen phosphate is not as reactive as hydrogen phosphate or phosphate ions with calcium ions [106]. Therefore, pattern formation at the outer electrolyte pH of 5.5 was not observed at different gel pH (**Figure 28**).



When the pH of the outer electrolyte was adjusted to 7.0, dihydrogen phosphate was again the dominant phosphate species; yet, the solution also contained some amount of hydrogen phosphate species. Thus, patterns still formed. pH values of 9.0 and 10.5 were ideal for Liesegang patterning, as the dominant species is hydrogen phosphate. pH 9.0 contained a limited amount of phosphate, whereas the amount of phosphate ions increased at pH 10.5. That is why both pH values resulted in relatively successful pattern formation (**Figure 28a-b**).

The pH of the gel should be considered in macroscopic patterning as well. At gel pH 4.0, carboxylate groups are mostly in their protonated form, leaving the gelatin positively charged due to the guanidinium group. In such a situation, the outer electrolyte with pH 5.5 cannot form any precipitate. Some of the reasons for the absence of precipitation might be calcium ions being free and soluble, resultant complexes being in soluble forms, and further protonation of phosphate species due to a highly acidic gel environment. The latter reason may also be the cause of relatively weak pattern formation at different outer electrolyte pH (**Figure 28a**). Even though the patterns formed, random precipitates were observed in each outer electrolyte pH, strongly implying that the gel environment led to the protonation of phosphate species. When both gel and outer electrolyte concentrations were set to pH 5.5, random precipitation took place (**Figure 28b**). The gel pH of 5.5 means that carboxylate groups are partially protonated and can still interact with some of the calcium ions, whereas the outer electrolyte pH of 5.5 implies most phosphate species are dihydrogen phosphate. Under these conditions, it is expected to have a lower supersaturation level, restricting periodic band formation. The pH of the outer electrolyte increased to 7.0, leading to the formation of more intact-fin bands rather than split-fin bands

observed in different systems. pH 9.0, on the other hand, resulted in the formation of regular split-fin bands due to the abundance of hydrogen phosphate ions. At pH 10.5, the bands become slightly wider and more diffused.

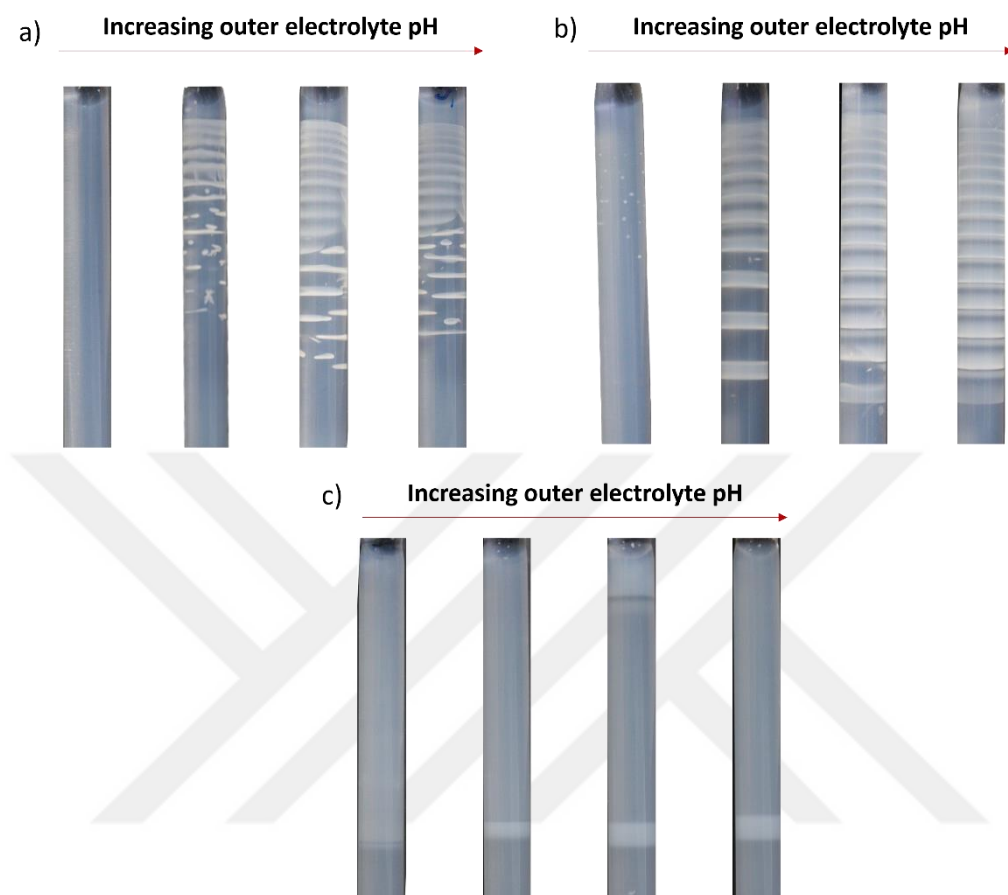


Figure 28. CaP LP samples produced under different outer electrolyte and gel pH conditions. pH of the gel is a) 4.0, b) 5.5, and c) 12. pH of outer electrolyte solutions is 5.5, 7.0, 9.0, and 12.

The gel pH of 12 resulted in continuous precipitate and only a sharp front after a continuous precipitation region at all outer electrolyte solution pH conditions (**Figure 28c**). pH 12 means that most of the calcium ions had already formed calcium hydroxide precipitate (due to the use of sodium hydroxide to adjust pH). That is why phosphate saturation might not have been enough to produce patterns, but a continuous region formed instead. Also, a high pH of the gel means a higher ionic strength, which may decrease gel porosity and make the gel more rigid, hindering phosphate diffusion.

Microscopic properties of precipitates showed striking differences, too. When the gel pH was 4.0, most of the carboxylate groups were in their protonated form, making them less susceptible to acting as nucleation sites. Therefore, fewer particles formed compared to the pH 5.5 gel environment. These particles remained loosely aggregated even if they created ordered

nanoflower structures. Using a pH 7.0 outer electrolyte solution led to the formation of flake-like morphology, loosely aggregated-crumpled morphology, and small nanoflowers (**Figure 29**), which can be attributed to the local conditions of the gel microenvironment. A similar trend was observed at pH 10.5 in terms of particle shape. Although the particle shapes remained similar, the number and size of particles were slightly different at pH 7.0 and 10.5 (**Figure 29**). As stated previously, dihydrogen phosphate with hydrogen phosphate ions and hydrogen phosphate with increasing phosphate ion concentration are the dominant phosphate species at pH 7.0 and 10.5, respectively. A highly acidic gel environment might have protonated highly reactive phosphate ions, leading to a decrease in nucleation. Therefore, fewer particles could form. pH 9.0, on the other hand, was the optimum pH for pattern formation as it mostly consisted of hydrogen phosphate ions. In that regard, it could form fewer nucleates and lead to the formation of larger crystals (**Figure 29**). Random precipitates also exhibited several morphologies, including agglomerated-crumpled, sheet-like, and nanoflower structures. This can be attributed to the varying supersaturation levels and local pH microenvironments in which particles formed.

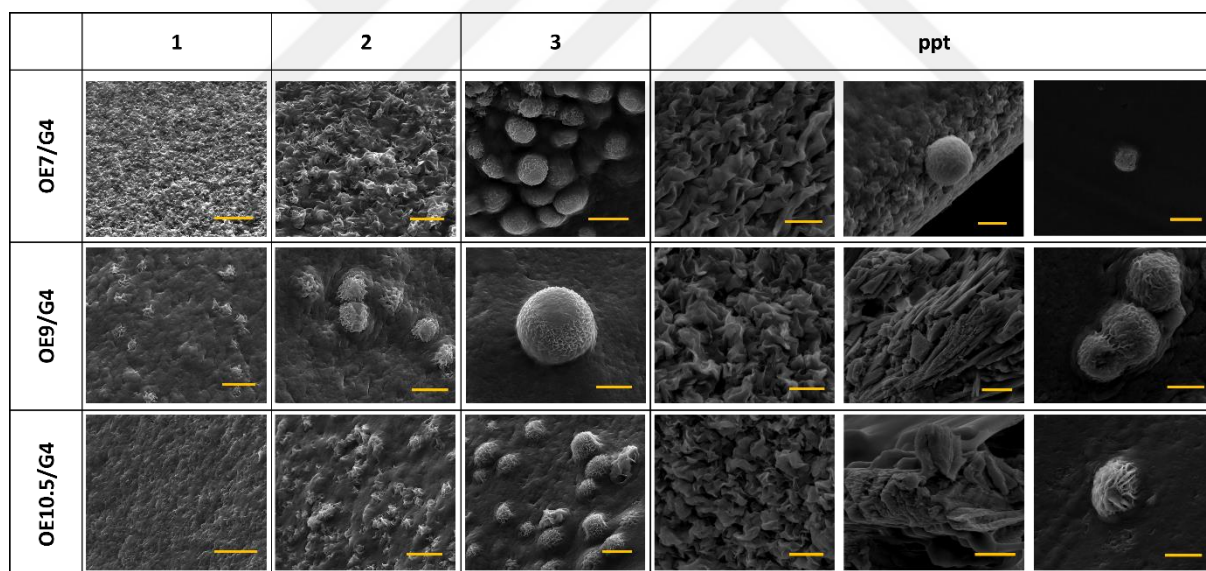


Figure 29. Particle morphologies of CaP samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin gel with a pH of 4.0 and varying outer electrolyte concentrations. (Please refer to Section 2.4 for abbreviations.) Numbers 1, 2, and 3 symbolize different parts of the same band, and ‘ppt’ denotes random precipitates formed after the LP bands. The scale bar is 5 μ m for OE7/G4-2, OE7/G4-ppt1&2, OE9/G4-ppt1, and OE10.5/G4-ppt1, and 10 μ m for the remaining micrographs.

In the case where gel pH was unaltered (5.5), only outer electrolyte pH varied through experiments, and the outer electrolyte pH was set to 5.5; only random precipitates with small cubic particles were obtained instead of periodic precipitation bands (**Figure 30**). Relatively lower supersaturation levels and slower reaction of dihydrogen phosphate ions might have suppressed band formation and resulted in random precipitates. These factors may also have contributed to the resultant particle morphology. In **Figure 30**, the second-to-last bands were investigated; however, this band corresponded to the intact-fin band at outer electrolyte pH 7.0. Therefore, both this band and the last-forming split-fin bands were analyzed. The crystal shapes remained the same across different outer electrolyte pH, even though the density and size of the crystals varied slightly. Comparing this system with samples having a gel pH of 4.0, the results strongly suggest that the gel pH has a prominent effect on both macroscopic and microscopic properties of CaP LPs.

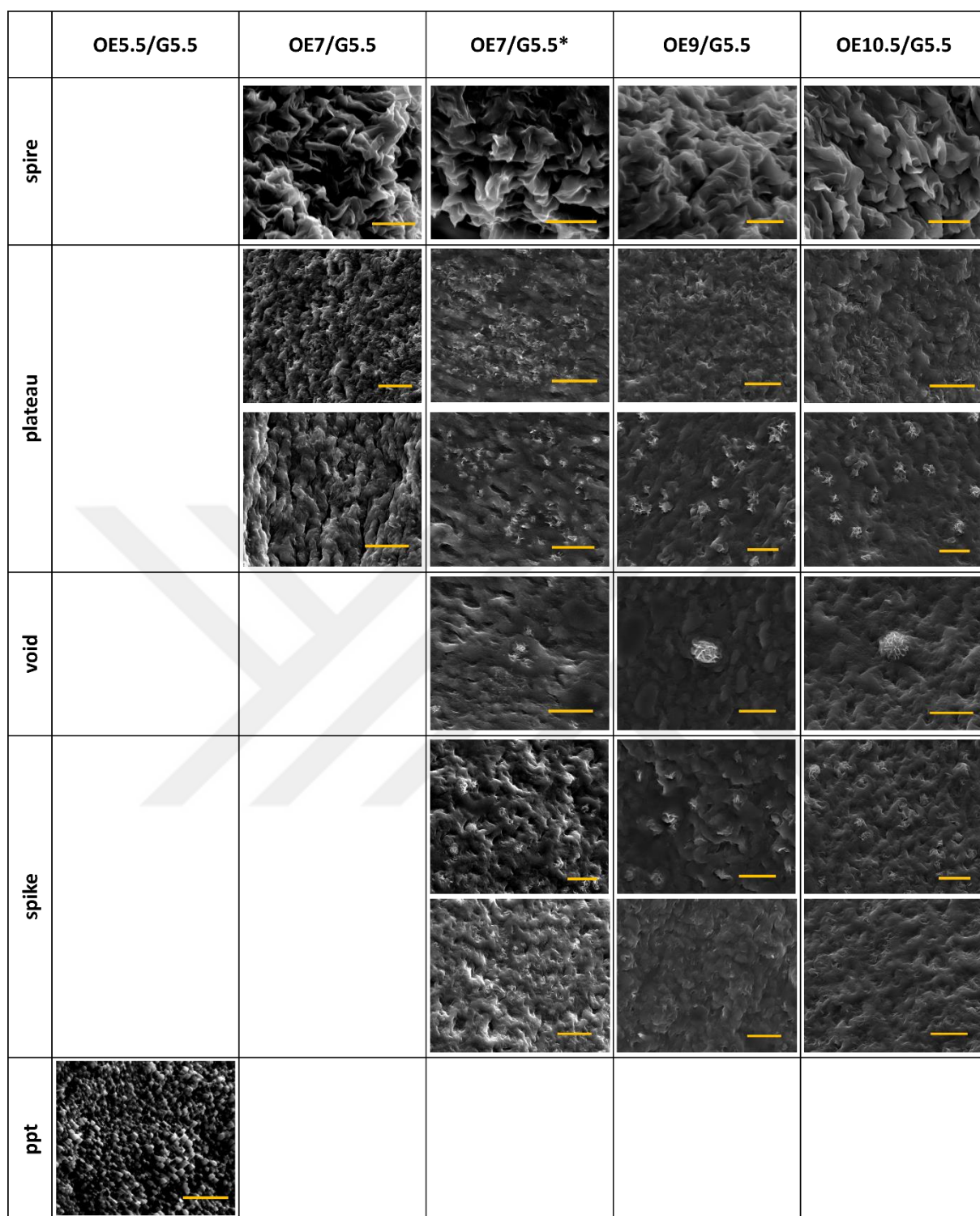


Figure 30. SEM micrographs of the product formed in CaP LPs prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium with pH 5.5. ppt stands for random precipitate. “*” means the last split-fin band of the OE7/G5.5 sample. (Please refer to 2.4 for abbreviations.) The scale bar is 5 μ m for spire and ppt regions and 10 μ m for the remaining images.

A basic gel environment (pH=12) was also used to produce CaP LPs. As stated, only continuous precipitation was observed in all samples. Ionic strength might have increased upon the addition of sodium hydroxide to basify the gel medium, leading to a more compact gel structure, which restricted the diffusion of ions. It can also be assumed that such a high pH might have altered the gelation behavior of blends. The addition of sodium hydroxide may have led to the instantaneous precipitation of calcium hydroxide even before gelation. This might have eliminated periodic banding and resulted in continuous precipitate. EDX analysis has confirmed that the crystals included an appreciable amount of phosphorus ions, meaning that possibly calcium hydroxide and phosphates co-precipitated or formed mixed-phase particles that were detected under SEM. Nanometer-scaled particles were obtained under different outer electrolyte pH conditions (**Figure 31**). This could be a result of rapid nucleation due to the addition of hydroxide ions. As carboxylate groups would be deprotonated at this pH, observing many nuclei samples was expected. The particles in the sharp front were relatively larger compared to the continuous precipitation region, which can be attributed to the diffusion length.

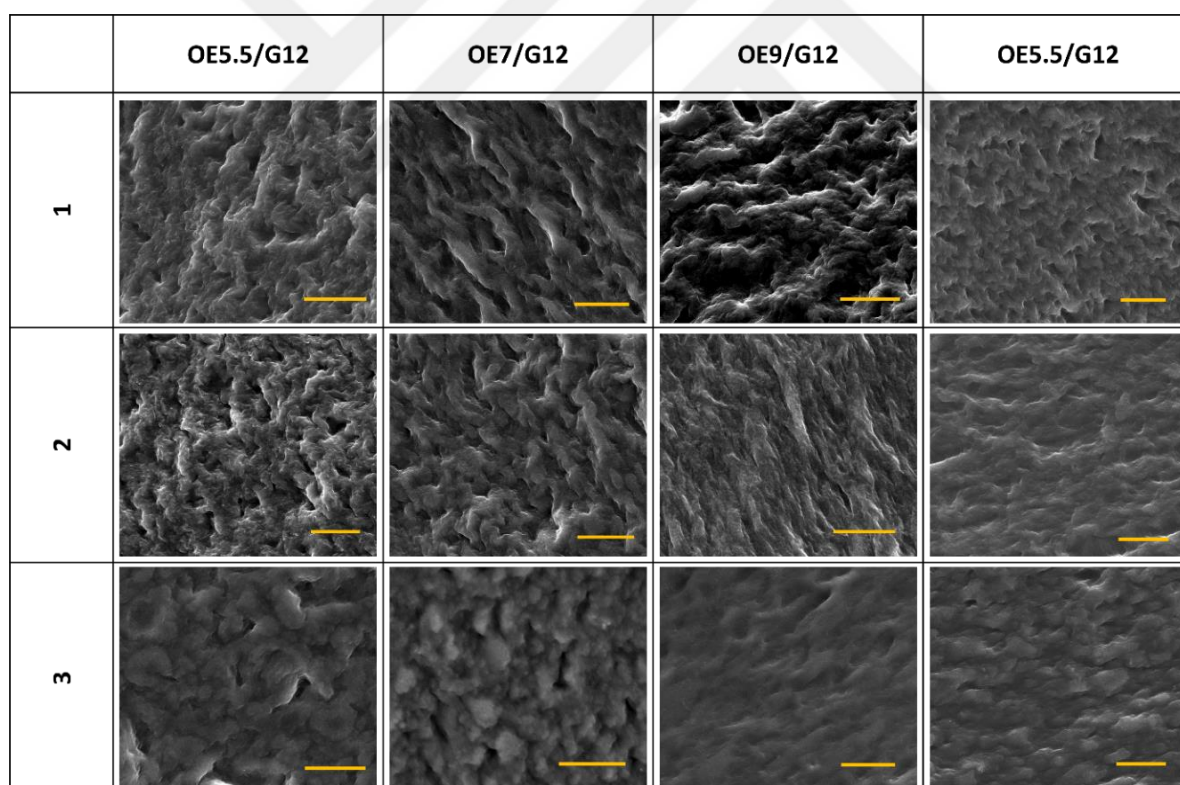


Figure 31. SEM micrographs of the product formed in CaP LPs prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium with pH 12. 1 and 2 represent different regions of continuous precipitate, and 3 corresponds to the sharp front forming after the continuous part (Please refer to 2.4 for abbreviations.) The scale bar is 10 μm .

Tables 6 and 7 display the Ca/P ratio of samples produced under different outer electrolyte and gel pH conditions. While outer electrolyte pH does not seem to change the ratio in a meaningful way, alterations in pH of the gel resulted in variations in this ratio. Particularly, when the pH of the gel was 12, the ratio was slightly higher than that of gel pH 4.0 and 5.5. It might have originated from the promotion of basic calcium phosphates (e.g., HAp) by the introduction of hydroxide ions with sodium hydroxide addition. The presence of hydroxide ions may have triggered the precipitation of hydroxyapatite-rich phases.

Table 6. Ca/P ratio of different bands of samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium under different gel and outer electrolyte pH conditions. 1, 2, and 3 symbolize different bands (or regions in the case of gel pH 12), while ppt stands for random precipitates formed after bands. (Please refer to **2.4** for abbreviations.)

	1	2	3	ppt
OE7/G4	1.10 - 1.27	1.16 - 1.39	1.02 - 1.23	1.16
OE9/G4	1.06 - 1.21	1.05 - 1.31	1.13 - 1.39	1.14 – 1.41
OE10.5/G4	1.17 - 1.25	1.22 – 1.38	1.31 - 1.39	1.17 - 1.57
OE5.5/G12	1.35 - 1.47	1.21 - 1.59	1.33	
OE7/G12			1.30 - 1.58	
OE9/G12	1.44 - 1.51	1.18 - 1.31	1.12 – 1.39	
OE10.5/G12	1.30 – 1.54	1.26 - 1.36	1.37 - 1.50	

Table 7. Ca/P ratio of different bands of samples prepared in 3.0% (w/v) agarose + 2.0% (w/v) gelatin medium under different outer electrolyte pH conditions. ‘ppt’ stands for random precipitates formed. (Please refer to **2.4** for abbreviations.)

	spire	plateau	void	spike	ppt
OE5.5/G5.5					1.01 – 1.14
OE7/G5.5	1.18 – 1.39	1.18 - 1.34			
OE7/G5.5*	1.47		1.07	1.07 – 1.17	
OE9/G5.5	1.18 – 1.42	1.11 - 1.55	1.46 - 1.50	1.24 - 1.54	
OE10.5/G5.5	1.16 - 1.20	1.18 - 1.45	1.14 - 1.18	1.11 - 1.35	

3.3.4 The Effect of Functional Groups on Morphology

The results displayed above imply that the changes in morphology stemmed from the presence of functional groups in the structure of gelatin, acting as possible nucleation sites and shaping the minerals that form. To display the drastic effect of the gelatin presence in the mineral formation, a set of identical samples with only 0.018 % (w/v) gelatin included in the 1.0% (w/v) agarose was prepared. In such an environment, the concentration of gelatin was 56 times lower than that of agarose; therefore, it can be considered an impurity. It was observed that both macroscopic and microscopic properties of the CaP formed were influenced by the inclusion of such a small amount of gelatin. In gelatin-containing agarose medium, both the dendritic particles and loosely-bound aggregates/nanoflowers were detected (**Figure 32b**), whereas in only agarose medium, only loosely-bound nanoflower structures (**Figure 32a**) were observed. The continuous precipitation region also had differences in particle morphology (e.g., size, number, and shape) when gelatin was included. However, the changes in this region were less apparent compared to the bands. As claimed, functional groups on gelatin acted as the nucleation sites and resulted in the formation of more nuclei, and thus, together with the nanoflower structures, dendritic structures could be formed.

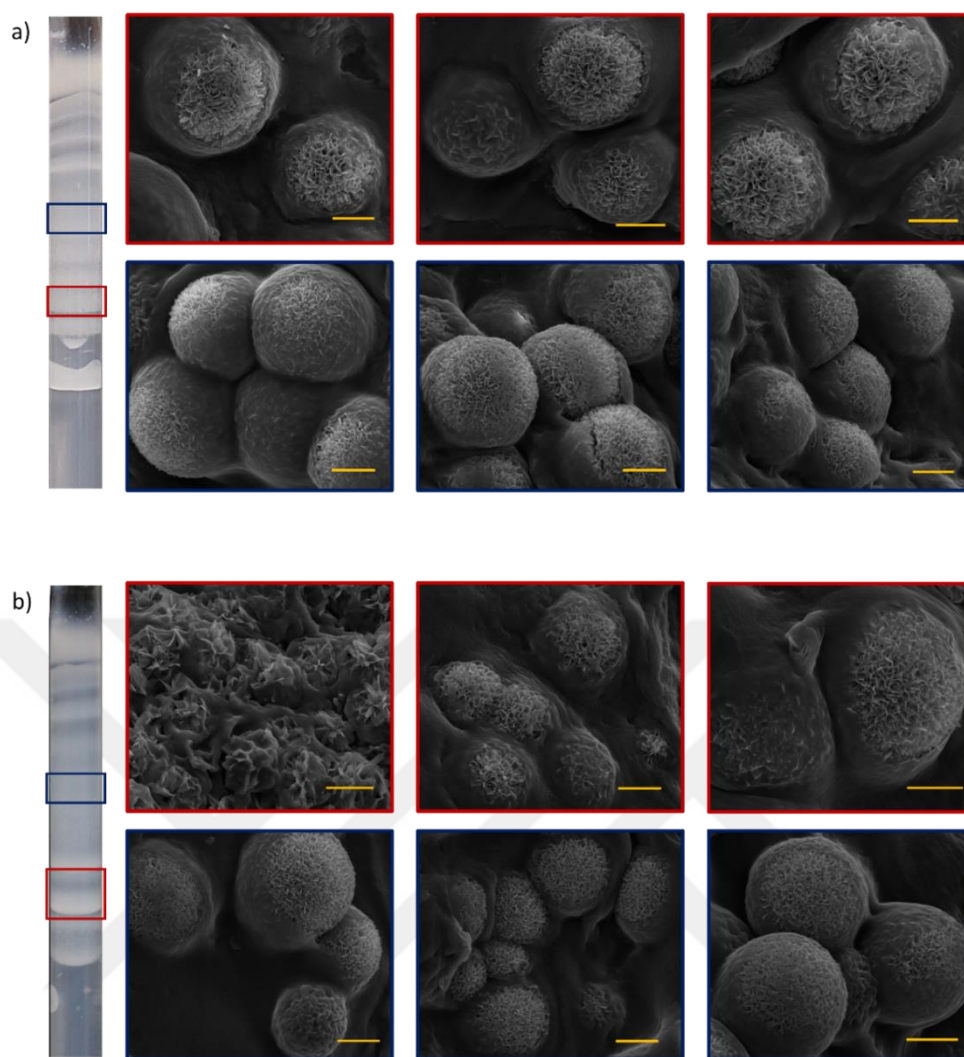


Figure 32. Photos and SEM micrographs of the product in CaP LP samples prepared in a) 1.0% (w/v) agarose, and b) 1.0% (w/v) agarose + 0.018% (w/v) gelatin media. Red-framed and blue-framed regions display the LP and continuous precipitation regions, respectively. The scale bar is 10 μm .

In addition to the SEM micrographs that allow for the visualization of an increasing number of nucleation centers in the presence of gelatin, infrared spectroscopy can be used to display the specific interactions of the ions with the polymers in the gel. ATR-IR spectra of the gelatin and agarose gels and their calcium and phosphate-ion-added forms were compared. In gelatin, the peaks at $\sim 1628\text{ cm}^{-1}$ and $\sim 1395\text{ cm}^{-1}$ correspond to stretching of C=O and symmetric stretching of COO^- groups [107], respectively. When calcium ions were present in gelatin, no change was observed for the stretching mode of C=O, which might have been a result of the overlap of this band with the amide II region of gelatin. However, a blue shift to 1404 cm^{-1} was detected in the symmetric stretching mode (**Figure 33a**). This shift can be interpreted as a result of a stronger C-O bond upon calcium ion interaction. C=N stretching

vibrations appearing at 1628 cm^{-1} [107] were investigated for phosphate interaction, too. The spectra showed no significant change in this region when phosphate was diffused into the gel. Thus, there is no spectral evidence of electrostatic interactions between phosphate ions and the guanidium groups in gelatin (**Figure 33a**).

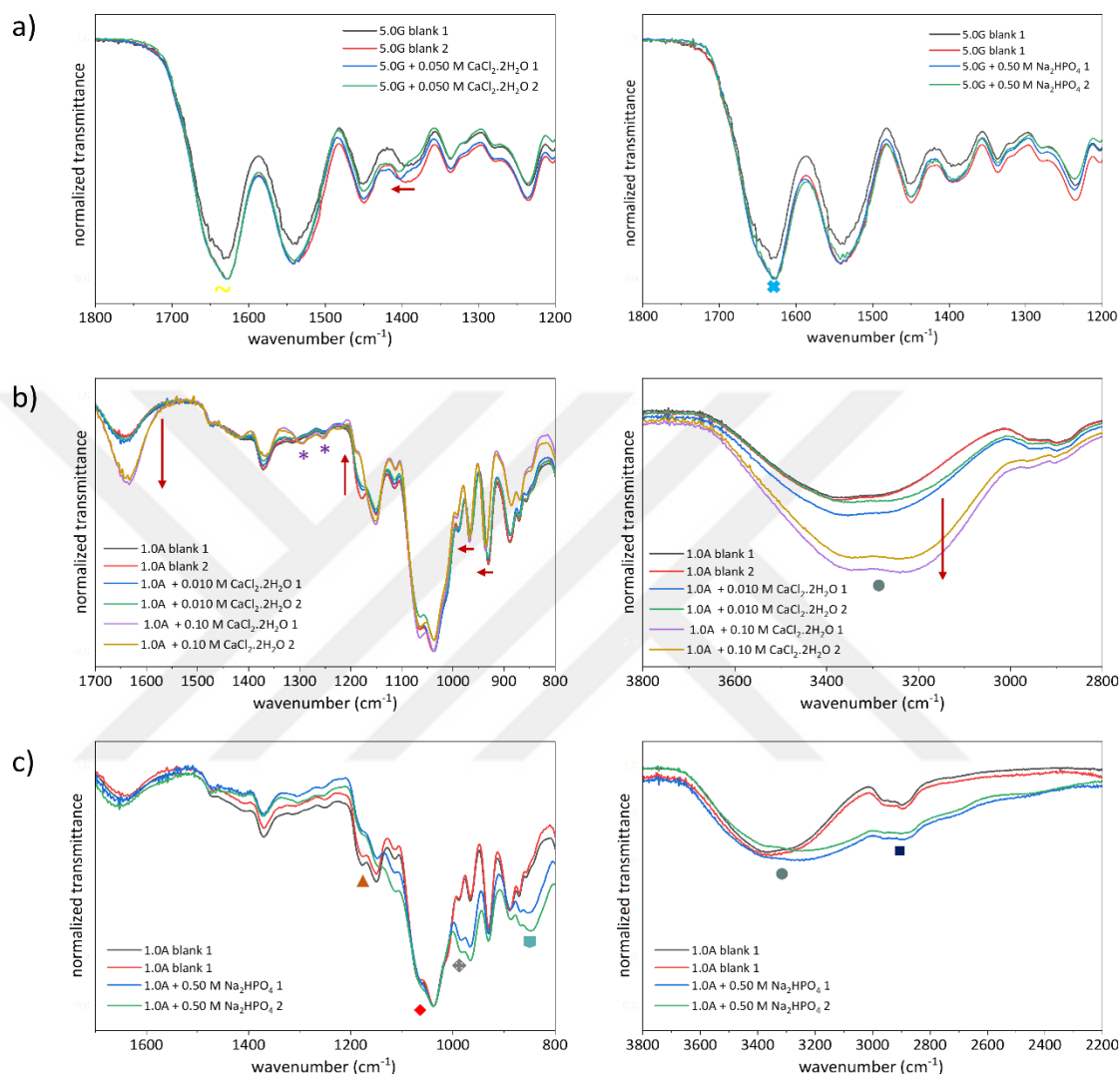


Figure 33. The IR spectra of a) gelatin samples either doped with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ or prepared with diffusion of Na_2HPO_4 in gel medium, b) agarose samples doped with different concentrations of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and c) agarose samples prepared with diffusion of Na_2HPO_4 in gel medium. A and G correspond to % (w/v) agarose and % (w/v) gelatin, respectively. Any changes or regions expected to have changes are marked with either arrows or different symbols.

In agarose, no significant change was expected in the IR spectra with the addition of the ions. However, both phosphate and calcium introduction to agarose led to a series of changes in the IR spectra of samples (**Figure 33 b-c**). Decreases or increases in the peak intensity, peak broadening, and shifts were observed after ion addition. This can be attributed to either physical

interactions between agarose and ions or conformational changes in agarose structure (especially backbone) due to the presence of ions.

3.4 CaP LPs in Agarose-Collagen and Gelatin-Collagen Blends

As previously stated, the extracellular matrix of bones contains protein (collagen), non-collagenous proteins (osteocalcin), polysaccharides (glycosaminoglycan), and inorganic minerals (hydroxyapatite) [108]. Gelatin-collagen and agarose-collagen blends were used to mimic the ECM better, as the protein part mostly consists of collagen. In our experiments depicted in **Figure 34**, gelatin-collagen blends form successful Liesegang patterning, while agarose-collagen blends do not lead to the formation of periodic precipitation.

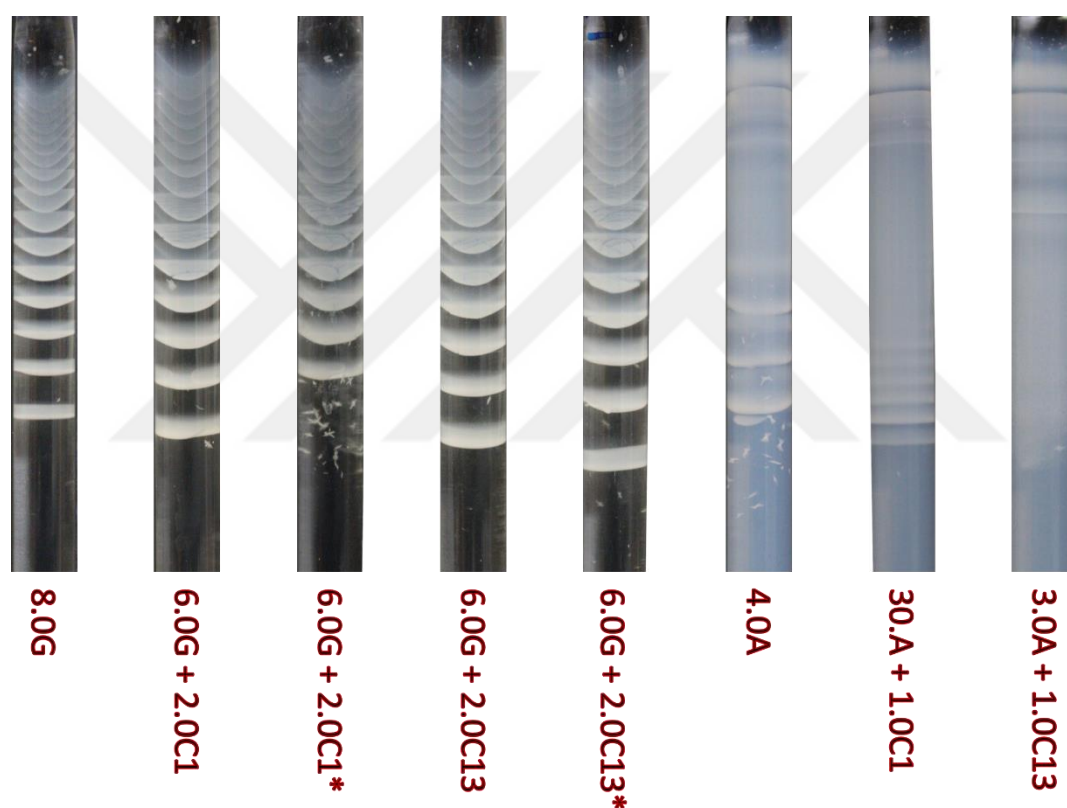


Figure 34. CaP LPs generated in agarose and gelatin control samples, and gelatin-collagen and agarose-collagen blends. G, A, C1, and C13 stand for % (w/v) gelatin, % (w/v) agarose, % (w/v) type I collagen, and % (w/v) type I&III collagen, respectively. “*” symbol represents that calcium is doped to collagen instead of gelatin.

3.4.1 Pattern Formation in Gelatin-Collagen Blend Systems

The CaP LP formed clearly in gelatin-collagen blends. The LP bands formed only two distinct regions, spire and plateau, contrary to what has been observed in agarose-gelatin blends. The spire and plateau regions displayed the same particle shape in both gelatin and gelatin-

collagen blend samples (**Figure 35**). The spire region consisted of large agglomerated-crumpled particles, whereas smaller flake-like crystals, which were “buried” inside gel in some cases, formed in the plateau across all samples. Except for slight variations in density and size of crystals, all blends produced similar crystal shapes. Considering that gelatin is a thermally denatured form of collagen with shorter polypeptide chains, the absence of alterations in crystal shapes was an expected result. Slight changes in size and crystal density can be attributed to the structural differences between gelatin and different types of collagen networks.

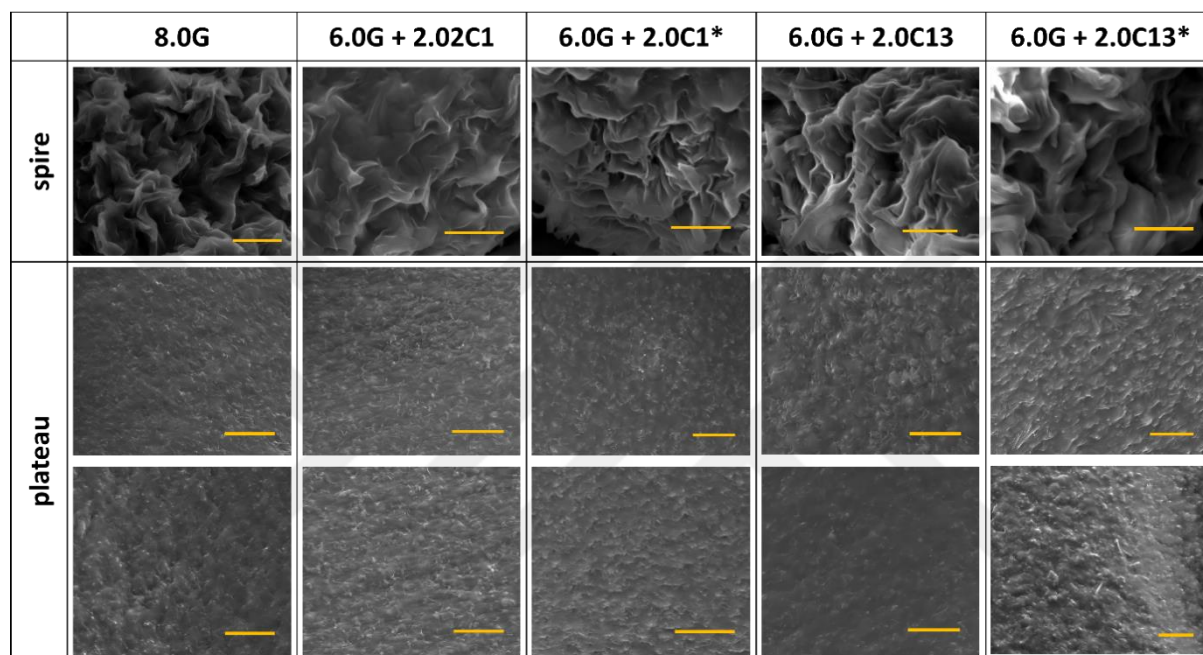


Figure 35. SEM micrographs of the product formed in CaP LPs prepared in gelatin-collagen blends. G, C1, and C13 stand for % (w/v) gelatin, % (w/v) type I collagen, and % (w/v) type I&III collagen, respectively. “*” symbol represents that calcium is doped to collagen instead of gelatin. The scale bar is 5 μm for spire regions and 10 μm for plateau regions.

3.4.2 Pattern Formation in Agarose-Collagen Blend Systems

The CaP LP formation was not as successful as agarose-gelatin blends (**Figures 14 and 34**) in the agarose-collagen systems. Although a few bands formed before and after the continuous precipitation region, they lacked the separate band regions that were observed in agarose-gelatin blends. Agarose control samples exhibited loosely-associated nanoflower structures regardless of the region that was examined. Agarose-collagen blend samples, on the other hand, displayed loosely-associated aggregate and dendritic morphologies when type I collagen was utilized. The use of type I&III collagen resulted in flake-like, dendritic, and loosely-bound nanoflower structures (**Figure 36**). The continuous precipitation region of this

sample also possessed loosely-bound but much larger nanoflowers. It has been observed that the number of particles increased upon blending with collagen, implying that functional groups of the collagen -similar to those in gelatin - acted as nucleation centers.

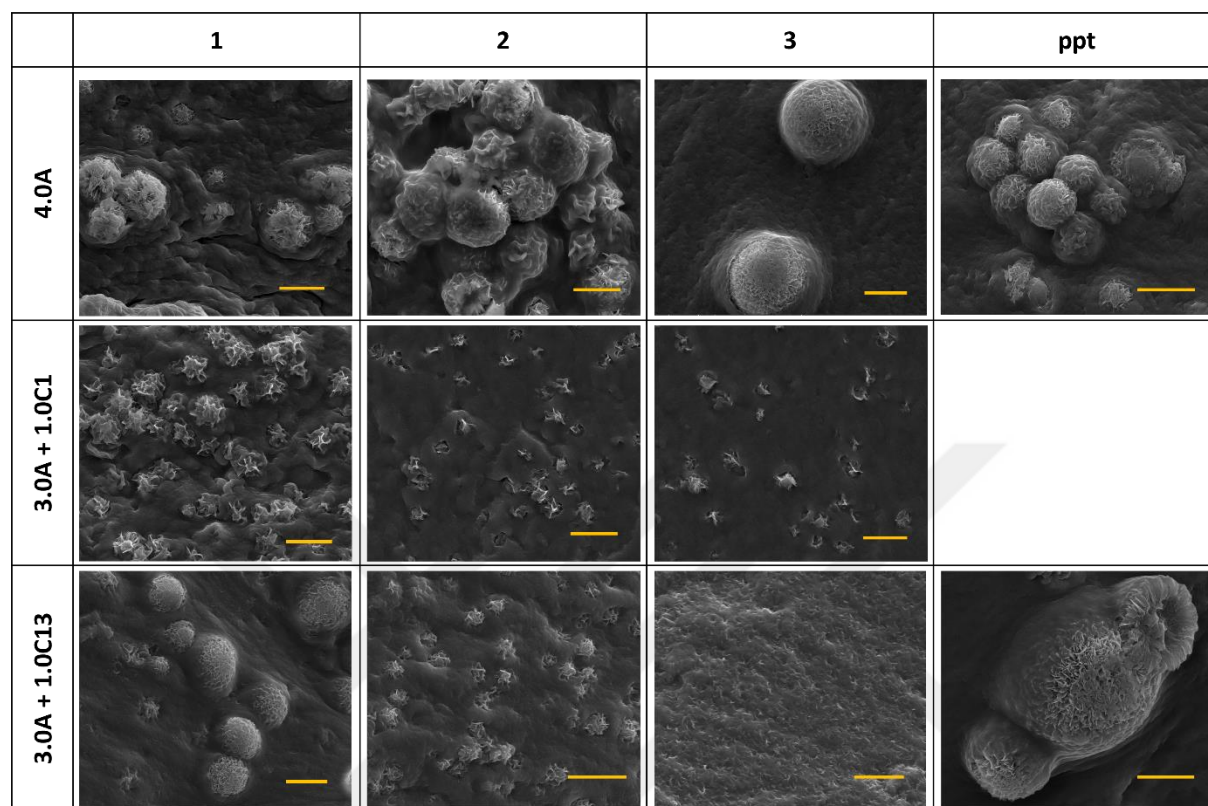


Figure 36. SEM micrographs of CaP LPs produced in agarose-collagen blends. A, C1, and C13 stand for % (w/v) agarose, % (w/v) type I collagen, and % (w/v) type I&III collagen, respectively. “ppt” stands for continuous precipitation region in the middle of the samples. The scale bar is 10 μm .

3.5 Potential Applications of CaP LPs in Blend Samples

Biocomposites consisting of calcium phosphates and hydrogels are presented as promising candidates for next-generation biomaterials due to enhanced properties such as high porosity, osteoconductivity, and variant morphological features. However, most studies lack successful biomimicry, as these studies have mostly been conducted in simple laboratory settings without consideration of the complexity of the natural biomineralization process. The other concern of these works is the use of chemicals, such as methacrylate, leading to cytotoxic effects. In this work, the properties of more complex patterned composite hydrogels were investigated by using protein-polysaccharide-calcium phosphate blends. The combination of Liesegang patterning with biomineralization resulted in the formation of varying morphological features in a hierarchical manner, making it a proper candidate for bone-regenerative materials.

Obtaining several morphologies in a small distance, like 3 to 4 mm, is making this system an even better candidate for use in a bioceramic material.

Due to experimental limitations, surface area analysis of the samples could not be performed, but the different morphologies displayed strongly suggest that the surface area of the crystals formed varied. It is among the most significant parameters that calcium phosphate particles have a high surface area and can be used as drug delivery systems for the controlled release of drug molecules. Varying surface areas and porosities of samples can make the drug release process even more controlled than previous materials used for this purpose. Also, incorporating agarose into gelatin (used often as a scaffold in drug delivery systems) prevents undesired swelling of the hydrogel, which leads to a mechanically weak structure, and uncontrolled release of drug molecules. Agarose adds thermal stability to the blend, too, which can also be advantageous for the same purpose.

The absence of toxic chemicals in the synthesis process makes this work valuable, as the use of certain chemicals leads to toxic effects, making composites unusable. In some situations, biocomposites may trigger an inflammatory response due to the use of certain chemicals. Inflammatory response can be classified as one of the biggest challenges of biocomposite design and might be limited or eliminated by choosing the right biomacromolecules. Our systems present a greener alternative to most studies conducted in this field, so far. Therefore, it can be reasonable to assume that the inflammatory response can be reduced with these materials that consist of only biopolymers. Nevertheless, it is necessary to perform *in vitro* experiments with cell cultures to confirm that the inflammatory response can be eliminated with these blends. In addition, SBF (stimulated body fluid) and PBS (phosphate-buffered saline) tests should also be considered to understand how the materials might behave *in vivo*. These tests are required to determine the bioactivity and physicochemical stability of composites. Although the overall results seem to be promising for these materials to be used in different fields, further *in vitro* and *in vivo* experiments are needed to confirm their availability as biocomposites.

CHAPTER 4

4. Conclusion

This work presents a new approach to the design of biocomposite materials by combining biomineralization and Liesegang patterns concepts, as well as to the understanding of the underlying mechanisms of biomineralization. Different hydrogel blend samples were prepared to produce optimum results for the periodic pattern formation, as the use of protein-polysaccharide complexes provides a better mimicry of natural systems' complexity. In this way, an enhanced biomimetic system was obtained. The first part of this study effectively demonstrated that not all Liesegang systems were capable of producing morphological variations when blend hydrogel samples were utilized. CaP LPs were, on the other hand, proper samples to achieve controllable particle morphology. With this study, it was shown that the control of particle morphology across different regions of a single band was possible. This work is the first study to show that within-band variety in an LP system is achievable. The observation that within-band variety is reproducible across different bands of the same sample makes the results even more valuable, as a hierarchical spatial arrangement of bands exhibiting intra-band variations has not been reported before. The crystal structure of precipitates was determined as OCP regardless of regions or bands. The samples prepared through the wet-chemical precipitation method confirmed that local concentrations of phosphate ions or local pH were not the only parameters in determining crystal structure and morphology, and the gel environment also had a crucial role.

Another significant result of this study is to have a robust system against experimental conditions. The samples prepared with different electrolyte concentrations produced macroscopic bands exclusively with slight deteriorations in the last-forming a few bands. Variations in the gel and outer electrolyte concentrations also resulted in successful macroscopic banding as well as consistent morphological differences across different regions of a band. These results strongly suggest that CaP LPs are robust against changes in ion and organic component concentrations, making them better candidates for biomimetic pattern formation, as these parameters dynamically change in natural processes.

This study also includes the effects of temperature and pH on macroscopic patterns and their microscopic properties. It was concluded that lower temperatures favored uniform morphology across a band. The reaction kinetics and diffusion were more dominant at higher temperatures. Thus, more and larger crystals were obtained. The effect of pH was more

prominent as it was directly correlated with phosphate speciation and the availability of functional groups as nucleation centers. Lower outer electrolyte pH resulted in the formation of relatively unreactive dihydrogen phosphate species, making it harder to form macroscopic patterns, while higher pH promoted phosphate ions. Lower gel pH, on the other hand, protonated carboxylate groups, and led to the availability of fewer nucleation sites. Rapid nucleation and possibly co-precipitation of calcium hydroxide and calcium phosphate were the results of higher gel pH conditions, which formed only a continuous precipitation region. Temperature and pH-related experiments verified that both pattern formation and biomineralization processes are a successful combination of several parameters, including diffusion, thermodynamic favorability, and supersaturation conditions. Therefore, the robustness of this system against concentration variations supports the presence of a highly stable order.

This work also shows that the interactions of ions with the gel structure have a determinative role in crystal morphology. SEM analysis displayed that even a minute amount of gelatin addition altered the particle morphology, while IR studies confirmed electrostatic interactions between calcium ions and carboxylate groups in gelatin and structural changes of agarose and/or possible interactions of it with ions. Considering the presence of reproducible minimal heterogeneity in blend samples, it might be reasonable to assume that CaP particles may be forming in a blend system in which the local gel microenvironment is the reason for hierarchical within-band variations.

The last part of the study consisted of collagen blends of gelatin and agarose. Gelatin-collagen blends produced successful macroscopic banding, while crystal shapes were not affected by the blending process. On the other hand, agarose-collagen blends formed different crystal shapes with varying sizes but failed to generate periodic patterns. Therefore, agarose-gelatin blend was determined to be the optimum system to produce CaP LPs.

Overall, this study examines several parameters in forming CaP LPs. The main focus was to investigate morphological features of precipitates, as crystal morphology plays a significant role in material properties. Using blend hydrogels introduced complexity, which also resulted in morphological variations without the requirement for toxic chemicals. The hierarchical order of bands makes these samples a candidate for next-generation biocomposites, while the variations resulting from experimental conditions might pave the way to understanding the underlying mechanisms of biomineral formation and adaptation. Therefore, the main purposes

of this study were met successfully. Future studies might explore the in vivo and in vitro activities of these composite gels for their potential applications in biomedical systems.



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