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The role of temperature in forming sol-gel biocomposites containing polydopamine

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Abstract

To further improve the physical strength and biomedical applicability of bioceramics built on hydroxyapatite-gelatin (HAp-Gel) and siloxane sol-gel reactions, we incorporated mussel adhesive inspired polydopamine (PD) into our original composite based on HAp-Gel cross-linked with siloxane. Surprisingly, with the addition of PD, we observed that the processing conditions and temperatures play an important role in the structure and performance of these materials. A systematic study to investigate this temperature dependence behavior discloses that the rate of crosslinking of silane during the sol-gel process is significantly influenced by the temperature, whereas the polymerization of the dopamine only shows minor temperature dependence. With this discovery, we report an innovative thermal process for the design and application of these biocomposites.

Keywords:

Nanocompsite, Hydroxyapatite, Gelatin, Silicate, Osteogenesis

Introduction

The use of hydroxyapatite-gelatin (HAp-Gel) and siloxane sol-gel reactions have been extensively studied for bioceramic applications in the past decades.[1] Our previous study has demonstrated the ability of HAp-Gel to provide a suitable substrate for osteoblast attachment, growth, and subsequent osteogenesis.[2] These composites, however, were shown to require cross-linking from bis[3-(trimethoxysilyl)-propyl]ethylenediamine (enTMOS) to increase their strength,[3] and were thus termed as HAp-Gemosil (i.e., hydroxyapatite–gelatin modified with siloxane). The HAp-Gemosil composites successfully showed improved compressive strength and processability without sacrificing biocompatibility or osteoconductivity; however, the inclusion of the enTMOS network resulted in composites which were still too brittle for *in vivo* use. Prior work with bioceramics has shown that the incorporation of polymer chains into the ceramic matrix can help improve mechanical properties and strength of these composites, as well as *in vivo* and *in vitro* biocompatibility.[4] This trend was also observed in a modified HAp-Gemosil composite utilizing a biodegradable polymer based on *L*-lactide to improve the flexural strength.[5] Though the flexural strength was indeed increased, the polymer toughened HAp-Gemosil composites were plagued by poor adhesion between the hydrophilic HAp-Gel and the hydrophobic polymer, leading to a loss in the compressive strength. Good adhesion between components of a blend is necessary in order to maintain good mechanical properties and avoid creating internal loci which are prone to mechanical failure.[6] In short, the results obtained by investigating HAp-Gemosil and similar composites with polymers included highlight the need for alternative polymers and cross-linking routes, in order to further improve important application related properties of these composites.

Recently, investigations into the adhesive properties of several sessile marine organisms have identified a series of proteins from *mytilus edilus* which demonstrate remarkable adhesion to a wide range of substrates.[7] Inspired by fact that these proteins are rich in 3,4-dihydroxy-L-phenylalanine (DOPA) and lysine amino acids (amine), Messersmith and co-workers hypothesized that dopamine, a small molecular that contains both functionalities (DOPA and amine), could structurally mimic these complex proteins and offer similar adhesion behavior. Indeed, the same authors experimentally confirmed that dopamine undergoes self-polymerization under basic conditions to form polydopamines (PD),[8] which can form thin polymer coatings adhering to a variety of organic and inorganic materials. Similar polymerization behavior of dopamine and the excellent adhesion of PD to numerous materials have been further demonstrated by others,[6] though the structure of PD is not well understood.[9, 10] Since composites utilizing PD are formed from a homogenous dispersion containing dopamine, this polymerization can potentially introduce a new interconnected polymer network. This newly formed polymer network can serve to connect distal portions of the composite, thereby increasing long range interactions within a material. The combination of forming a polymer and increasing adhesion can potentially increase both long and short range interactions within a composite. For these reasons, the complex behavior of PD, and its numerous potential applications have become an attractive topic of study when investigating new materials for bioceramics.[11, 12]

Inspired by the exciting discoveries and features of polydopamine (PD), we attempted to introduce dopamine into our HAp-Gemosil formulation, and re-named these new composites as HAp-Gemosilamine (i.e., hydroxyapatite-gelatin modified with silane and dopamine). This type of composite was designed to impart the excellent adhesive properties of PD, the biocompatibility and osteoconductivity of hydroxyapatite, and the mechanical strength of the

enTMOS silsesquioxane network into a single phased composite. It was believed that the combination of these properties would yield strong, functional materials with possible future biological applications. A number of such HAp-Gemosilamine composites were then formulated and tested (**Figure 1**). Various processing conditions were utilized in order to thoroughly study the setting behavior of these materials. Interestingly, composites which were allowed to begin setting at depressed temperatures, – 20 °C, before finishing setting at 20 °C, showed markedly improved properties when compared with HAp-Gemosil. However, when these composites were processed entirely at 20 °C, their mechanical properties were surprisingly poor. The interesting discrepancies within HAp-Gemosilamine composites prompted us to conduct a careful investigation, which is detailed in this report.

Materials and Methods

2.1 Materials

HAp-Gel slurry was prepared according to the previous co-precipitation method developed by Chang *et al*.[13] The HAp-Gel slurries were freeze-dried at – 80 °C overnight followed by lyophilization until dry to form HAp-Gel powder. The $Ca(OH)_2$ was derived by hydration of CaO which was previously calcinated at 1250 °C for 3 hours.[14] enTMOS and dopamine·HCl were purchased from Gelest, Inc. (Morrisville, PA, USA) and Alfa Aesar, respectively.

2.2 Designing and Formulating HAp-Gemosilamine Composites

100 mg of HAp-Gel/HAp, 200 mg of $Ca(OH)_2$ powder, and 10 mg of dopamine·HCl powder were transferred into a mortar and ground into fine powder. For "cold" samples, the powder mixture was spread on a cold stage to maintain a depressed temperature of – 20 °C. In the cold stage, 383 μL of 62% enTMOS was added to the mixture while the powders and enTMOS were

continuously spatulated for 30 seconds. For "warm" samples, spatulating was done at 20 °C without utilizing the cold stage. After spatulating, 40 μL ammonium persulfate solution (1 M in PBS 1×) was added to the mixture to initiate the polydopamine reaction. At this state, the mixture became dark fluid, which can then be injected into a mold to create arbitrary-shaped samples. Once leaving the cold stage, the fluid solidified and stopped shape change within 3 minutes. The samples without dopamine were also made for comparison (denoted as HAp-Gemosil).

2.3 Compressive and Biaxial Flexural Testing

Cylindrical model with a 1:2 ratio of diameter (3.5 mm) to length (7.0 mm) was used to prepare compressive samples at two conditions: with and without cold stage mixing. All samples were dehydrated at the room temperature for 7 days. Compressive testing was performed on an Instron machine (model 4204, Canton, MA, USA) with a cross-head speed of 0.5 mm/min. The compressive strength was determined from the maximum strength value on the stress-strain curve. The testing procedure for the biaxial flexure strength was also performed according to methods established in our previous publication.[5] Briefly, disk samples (11 mm diameter by 2.7 mm thickness) of each group were prepared in Teflon molds. The upper and lower surfaces were polished in order to obtain parallel surfaces. A crosshead speed of 0.5 mm/min was used, and the maximum force at failure (P) was determined using an Instron machine (model 4411, Instron Co., Norwood, MA, USA). A Poisson's ratio of 0.3 was used and the flexure stress at failure was calculated. Five samples for each group were used in the above testing. The testing results were analyzed by one-way ANOVA.

2.4 Mass-Loss Experiments

Mass-loss experiments were conducted as follows. 200 μL of enTMOS was diluted and mixed thoroughly with 250 μL of a 95% v/v MeOH/H_2O solution in a pre-weighed 2 mL flask. This mixture was then allowed to set for a predetermined period of time. After this time, resulting material was rinsed 3 times with chloroform and 3 times with acetone. The residual solid remaining in the flask was then heated for 24 hours at 100 °C to remove any residual solvent. After 24 hours, the glassy material was again weighed to determine the % mass lost during the rinsing phase. For samples at – 20 °C, an identical approach was taken; however, all reagents were kept at – 20 °C for 24 hours before mixing. After mixing the pre-cooled reagents, the material was then stored at – 20 °C for the duration of the time interval until it was finally rinsed and heated as above.

2.5 UV-vis Experiments

Progress of dopamine oxidative polymerizations was monitored via UV-vis spectroscopy as follows. Dopamine (2 mL, 0.05 M in MeOH) was added to 1 mL of a saturated NaOH solution in MeOH. This mixture was probed with a UV-Vis spectrophotometer (model UV-2600, Shimadzu Scientific Instruments, Inc.) every 30 sec for 6 min at 350 nm. In order to investigate the reactions at – 20 °C, cold dopamine and NaOH solutions were mixed and stored in a controlled environment at – 20 °C. Again, measurements were taken every 30 sec for 6 min at 350 nm, and samples were kept cold until immediately prior to measurement.

2.6 Morphology Study

SEM (scanning electron microscopy) and TEM (transmission electron microscopy) analyses were done in order to further probe the morphology and phase distribution of HAp-Gemosilamine composites. For SEM analysis, 1 cm samples were cut from bulk HAp-Gemosilamine composites and imaged via a Hitachi S-4700 Cold Cathode Field Emission SEM

(Hitachi High Technologies America, Inc.). For TEM, the bulk HAp-Gemosilamine composite was ground into a fine powder and suspended in MeOH after sonication. One drop of this HAp-Gemosilamine solution was then added to the TEM grid for imaging via a JEOL 2010F-FasTEM (JEOL USA, Inc.).

2.7 Osteoblast Proliferation Assay

Preosteoblasts MC3T3-E1 was used to test viability of the materials in 35mm culture dishes. Cells were seeded at a density of 1×10^4 per milliliter using αMEM medium supplemented with 10% of FBS and 1% penicillin/streptomycin under 37 °C, 5% CO_2 atmosphere. Three groups were investigated, including HAp-Gemosil, HAp-Gemosilamine, and dishes as received without coating (control). The spin coating method was described in the previous reports[13,14] and the coated dishes were UV sterilized and dried for 7 days, which were soaked in 2 ml PBS overnight prior to cell seeding. After 1, 4, 7, 14, and 21 days in culture, 40 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) reagent (Promega, Madison, WI, USA) was added to each dish containing 400 μL of Alpha Minimal Essential Media (*α*-MEM, Sigma-Aldrich, St. Louis, MO, USA), and the dish was incubated for 1 hour at 37 °C under a humidified atmosphere of 5% CO_2. Each sample was triplicated by culturing three dishes and each dish was measured three times by transferring 100 μL of MTS solution into each well of the 96-well plate. The absorbance of each well at 490 nm was measured using a microplate reader (Microplate Reader 550, Bio-Rad laboratories, Philadelphia, PA, USA). Relative cell numbers were quantified on the basis of the concentration of the formazan product of MTS. Coated dishes with HAp-Gemosil and HAp-Gemosilamine without cells were used as a blank (background substrate).

2.8 3D Printing of HAp-Gemosilamine

A computer generated 3D cylindrical porous template (10 mm diameter by 6 mm height) was designed with the SolidWorks software (Dassault Systemes SolidWorks Corp., Waltham, MA, USA). This template (stl format) was then used to print a 3D wax mode made of Indura® Cast (Solidscape Inc., Merrimack, NH, USA) with $1mm^2$ trusses and $1mm^2$ pore size (continuous space) via a Solidscape 3D printer (Solidscape Inc., Merrimack, NH, USA). Next, the HAp-Gemosilamine mixture was prepared on the cold plate as described above and injected to fill up the pore spaces of the 3D mode while the material was in liquid form. The HAp-Gemosilamine in the 3D mode was set within 3-5 minutes at room temperature. Then the wax mode and HAp-Gemosilamine complex was immersed in acetone for 30 minutes to remove the wax template and release the 3D porous HAp-Gemosilamine scaffold, which is shown in Figure 6. The porous cylinders were air dried for one week and subjected to a compression test by an Instron machine with a compressive rate of 0.5 mm/min until the sample fractured. TestWorks 4 software (MTS Systems Corporation, Eden Prairie, MN, USA) was used to record the data and the compressive strength was determined from the maximum strength value on the stress-strain curve. The compressive strength of the porous scaffold was averaged from three samples.

Results and Discussion

3.1 Forming HAp-Gemosilamine Composites: Observation and Hypothesis

When dealing with these new HAp-Gemosilamine materials, it quickly became apparent that the processing temperature played a critical role in forming a robust composite capable of mimicking the mechanical strength found in natural bone. We denote the "warm" samples as the composite allowed to set at room temperature (20 °C) after mixing. The "cold" samples, on the other hand, were allowed to set for approximately 5 minutes at – 20 °C, before subsequent

warming to room temperature to finish the setting (**Figure 1**). While this difference in processing condition seems minor, the large difference observed in mechanical properties of the "warm" sample and the "cold" sample clearly demonstrates the opposite (**Figure 2**). For example, the HAp-Gemosilamine processed under the "cold" condition shows a compressive strength close to 100 MPa, noticeably higher than that of the HAp-Gemosil (~ 80 MPa), and more than doubling that of the HAp-Gemosilamine processed under the "warm" condition.

So what causes this interesting temperature dependence behavior? Since HAp-Gemosil composites did not show any processing temperature dependence on their material properties in our previous investigation, we believe that the newly added component, dopamine, must be playing a critical role in the observed temperature dependence of the mechanical properties of HAp-Gemosilamine. We hypothesize that while dopamine is able to polymerize uninterruptedly at both low temperatures and room temperature, the sol-gel reaction of silanes is significantly hindered at low temperatures. Therefore, when the mixture is allowed to react at – 20 °C, only polydopamine (PD) formation proceeds appreciably to form the PD network, whereas the sol-gel reaction of silanes only occurs to a negligible extent. However, the small molecule and liquid nature of the enTMOS molecules can permeate the entire composite even after the "soft" PD network is formed. When such a composite is allowed to warm up to room temperature, enTMOS starts to polymerize rapidly to form the silsesquioxane matrix that can effectively envelope the newly formed PD network, creating a series of inter-penetrating networks based on both PD and silsesquioxane. Such intricate networks formed by both PD and silsesquioxane help to strengthen the new composite, HAp-Gemosilamine, when compared with the original HAp-Gemosil composite. This "cold" processing, together with its effect on the formation and structure of the composite, is illustrated in **Figure 1a**. In contrast, when the entire composite is

processed at 20 °C, the rate of polymerization for enTMOS is overwhelmingly faster than that of the dopamine polymerization. Before dopamine can form an effective network via its own polymerization, the polymerization of enTMOS already yields a glassy, highly cross-linked silsesquioxane network. Such a "hard" network will trap dopamine molecules (and oligomers of PD) into isolated regions. This will eventually lead to *segregated* PD domains within the cross-linked silsesquioxane network, which is ultimately detrimental to mechanical properties of these composites. This scenario, i.e., the "warm" processing, is correspondingly illustrated in **Figure 1b**. In summary, we believe the difference in kinetics for these two reactions (i.e., polymerization of dopamine, and sol-gel cross-linking of silanes) and the mechanical nature of these two networks (i.e., soft gel of PD and hard glass of silsesquioxane network)would directly result in the significant difference in the micro/nano structures of these composites processed at different temperatures. Such structural differences in these composites lead to the observed dramatic difference in the mechanical properties, as shown in **Figure 2**. Specifically, the inclusion of PD in "cold" samples improves mechanical properties when compared with HAp-Gemosil materials, while the inclusion of PD in "warm" samples causes a dramatic reduction in mechanical strength.

3.2 Experimental Design: Practical Considerations

According to the hypothesis detailed above, investigating the reaction kinetics of each component individually should be the crucial step to experimentally elucidate their effect on final composite performance. This practice helps develop an understanding of how each component is able to work with the others to afford materials whose properties are greater than the sum of their parts. Such a further understanding would be important for optimizing and utilizing future PD containing bioceramic composites.

Ideally, one should investigate the polymerization behavior of one component (e.g., enTMOS) in the presence of the other component (e.g., dopamine) and other parts of the composites (e.g., HAp-Gel) to obtain the most relevant data. Unfortunately, within the newly developed HAp-Gemosilamine composite, several reactions are occurring among the various components of the material. This complication makes it very difficult to quantify the way a single component is behaving within the actual blend. Therefore it became necessary to simplify these reactions in order to more adequately characterize the behavior of a single component. For example, to further study how temperature can influence the progress of enTMOS sol-gel reactions, a series of experiments with enTMOS as the only component were carried out.

Similarly, to study the behavior of PD in the composite, it became necessary to alter the conditions under which PD was made to polymerize. When preparing HAp-Gemosilamine as stated above, PBS buffer, $Ca(OH)_2$, and Ammonium Persulfate (AP) were added to initiate cross-linking of enTMOS and subsequently PD. However, the actual concentration for dopamine used in HAp-Gemosilamine was too high to be observed via UV-Vis spectroscopy to monitor the reaction progress (*vide infra*). Therefore, we lowered the concentration of dopamine in solution (rather than in an almost solid state as in actual composite) such that the absorbance of the solution would be at the right level to track the progress of the reaction spectroscopically. Finally, because the concentration was lower, the freezing point would not be depressed in a manner similar to what we observed during processing of HAp-Gemosilamine. For this reason, it also became necessary to change solvents from water to methanol, otherwise the entire water based solution of such a low concentration would have been frozen at – 20 °C. This allowed our "cold" experiments to also be modelled in a manner similar to the way the "warm" samples were. Additionally, for solubility reasons, $Ca(OH)_2$ was replaced with NaOH and AP was not

used. AP was omitted in order to keep the reaction from happening too rapidly,[11] thereby allowing more time to monitor the reaction progress and to draw meaningful conclusions from our data.

Although these experiments do not exactly mimic the behavior and environment of enTMOS and dopamine within a composite, these data can help illuminate the observed inconsistencies between 20 °C and – 20 °C HAp-Gemosilamine samples.

3.3 Temperature Dependence of The Sol-Gel Reaction of enTMOS

Typically, enTMOS sol-gel polymerizations begin to cross-link and solidify within minutes after initiation at room temperature. Therefore, for a HAp-Gemosilamine composite at 20 °C, the sol-gel reaction of enTMOS occurs rapidly as methanol is removed and an insoluble, glassy composite remains. As a result, this brittle silsesquioxane matrix sets the composite within minutes, effectively blocking further appreciable molecular diffusion. The final weight of this glass is roughly 70% of the initial enTMOS loading, due to the loss of methanol through hydrolysis condensation reactions.

However, this sol-gel reaction of silanes has shown little spectroscopic difference as the reaction proceeds, making it difficult to spectroscopically track the reaction progress in the studied range of 20 °C and – 20 °C. Fortunately, the insolubility of this newly formed silsesquioxane gel allowed us to design a mass loss-based method to monitor the progress of this reaction. Specifically, by washing out the soluble portions of enTMOS (e.g., monomers and oligomers) at various time intervals, it would be possible to track how this reaction proceeds over time and determine what role temperature plays in this cross-linking polymerization. For example, if the reaction had not proceeded appreciably, nearly all of the initial enTMOS monomers and non-cross-linked oligomers would be washed away, showing a very high mass

loss. Conversely, if the reaction had been already in an advanced stage and highly cross-linked, almost no monomers and oligomers would be removed by washing, showing a very low mass loss. By determining the time point at which the cross-linking reaction begins and ends at suppressed and elevated temperatures, we can gain a better understanding on how the reaction is likely proceed within the much more complex composite (e.g., HAp-Gemosilamine).

Figure 3 presents the results from these mass loss experiments. These data clearly demonstrate that at – 20 °C, the sol-gel reaction enTMOS is retarded for tens of minutes, and these "cold" composites do not begin setting appreciably until after several hours. In contrast, this retardation is not observed for the same sol-gel reaction at 20 °C. Over the same time interval, the "warm" enTMOS composites proceed almost to completion. The significant dependence on temperature of this sol-gel reaction implies that when processing these composites with multiple components (e.g., HAp-Gemosilamine) at room temperature, the rapid polymerization of enTMOS would cause the composite to begin setting quickly at early stage. Afterwards, all reactions within the composite would become heavily diffusion controlled. This diffusion control can hinder the ability of other reactions to take place during the final setting of the composites. The interruption of these other reactions, such as dopamine polymerization, can lead to incomplete setting and poor mechanical strength. Apparently, lowering the temperature to slow down the sol-gel reaction of enTMOS would allow ample time for all desirable reactions to occur synergistically, resulting in a proper setting of the composite with improved mechanical strength.

3.4 Temperature Dependence of Dopamine Polymerization

Fortunately, unlike the enTMOS gelation, it is possible to use spectroscopy to monitor the progress of dopamine polymerization because the oxidation of dopamine to form the PD is

accompanied by a characteristic color change. As more monomers become oxidized and subsequently polymerize, the solution turns darker. This is likely caused by increased pi-pi interactions within the newly forming macromolecules. According to the work by Wei *et al*.,[11] the PD formation can be monitored using UV-Vis spectroscopy at 350 nm. This offers us a simple method for monitoring the formation of PD and is indicative of the extent of PD polymerization by comparing the intensity of the UV-Vis absorption at different time intervals. Though this does not give us a quantitative measure of dopamine's degree of polymerization, it gives us useful information about how this polymerization is progressing under a certain set of conditions.

In practice, we simplified the system to contain only dopamine, methanol, and NaOH. This simple system still retains the essence of the polymerization of dopamine to PD; more importantly, such a diluted system allows for visualizing and comparing the reactions progress in solution by the UV-Vis absorption. We then conducted these experiments for samples at both – 20 °C and 20 °C and the results are highlighted in **Figure 4.** It can be seen clearly that, while temperature does influence PD formation, this difference on reaction progress at each time interval is small between – 20 °C and 20 °C. Overall, these results support our hypothesis that the formation of PD is much less influenced by the temperature, showing significantly different reaction kinetics than that of enTMOS reactions (**Figure 3**).

In the new multi-component based composite – HAp-Gemosilamine, delaying the onset of the enTMOS gelation at low temperatures affords the composite more time for the other components to react more fully before being encapsulated within the new enTMOS silsesquioxane network and completely set. Specifically, the slowed gelation of enTMOS at low temperatures gives a brief time window where dopamine can polymerize relatively unhindered.

Though the true mechanism of the formation of PD is still under debate, the work by Hong *et al*.[9] suggests that the polymerization of dopamine could yield regions of trimers and tetramers which are able to stack through pi-pi interactions.[9] These small aggregates of trimers and tetramers can act effectively as weak cross-links between different PD chains, increasing the overall strength of the composite. After this initial cold setting phase, the composite is warmed up and enTMOS is able to then fully polymerize around the connected PD network, encapsulating it within a glassy silsesquioxane network. Therefore the adhesive ability of PD, and the subsequent formation of these interpenetrated polymer networks are key to the observed enhanced mechanical properties of the composite (**Figure 2**).

3.5 Morphology Study of HAp-Gemosilamine Composites

In order to probe the morphology and phase distribution of these newly formed HAp-Gemosilamine composites, various electron microscopy techniques were used. The SEM images (**Figure 5**) reveal the presence of two distinct phases: one is enriched with HAp-Gel, while the other is enriched with silsesquioxane and PD. The two phases were statistically homogenously dispersed throughout the HAp-Gemosilamine composite. The morphology and phase distributions suggest that, while the polymers exist in distinct phases, they are well blended with HAp-Gel. The end result is a highly interpenetrated polymer network incorporating HAp-Gel (as illustrated in **Figure 1)**, which leads to the unique mechanical properties of HAp-Gemosilamine in contrast to HAp-Gemosil.

Furthermore, TEM images reveal that after the extensive processing done for HAp-Gemosilamine composites, the HAp-Gel nanocrystals remain intact (**Figure 5**). This suggests that many of the favorable properties associated with the HAp-Gel platform may still indeed be intact within the HAp-Gemosilamine composite. These results demonstrate the improved

properties associated with incorporating PD into the original HAp-Gemosil composite, and highlight that many favorable aspects (e.g., maintaining HAp-Gel nanoccrystalline structures) of HAp-Gemosil and HAp-Gel are unaltered by the inclusion of PD into the composite.

3.6 Biocompatibility: MTS Assay

Though dopamine is found naturally in the human body, its properties as a bulk material outside the brain are not well understood. Because the new composite HAp-Gemosilamine showed improved mechanical properties than those of the original HAp-Gemosil under appropriate processing condition, it was important to verify that using PD as a component in HAp-Gemosilamine would not negatively impact the biological compatibility. MTS assays were then carried out to determine cell viability over a 21 day period. The growth of preosteoblasts (MC3T3-E1) on three variant surfaces was shown in **Figure 6**. Interestingly, during the first 5 days, the cells were sporadic and did not spread in HAp-Gemosil. In contrast, adding PD appeared to increase initial cell growth in the same period of time. After day 7, there were no statistical differences in cell numbers among three groups, indicating excellent biocompatibility of the new composite HAp-Gemosilamine and warranting further investigation.

3.7 3D Printed Scaffolds Formation and Testing

As detailed above, the reaction kinetics of HAp-Gemosilamine composites allows a brief time window during which the injected materials can fill arbitrary shapes, which can be very useful in tissue scaffolding applications. As a proof-of-concept, a simple scaffold was prepared by indirect 3D printing this material, as shown in **Figure 7**. Such porous scaffolds were further subjected to mechanical testing in order to probe how these materials would perform when processed into a scaffold. The compressive strength of the HAp-Gemosilamine scaffold with the pore size 1.0 mm was 15.86±0.96 MPa, which was comparable to that (1MPa -13 MPa) of

cancellous bone reported in the literature.[15] Our scaffold appears to provide adequate pore size for cell penetration and compressive strength for the replacement of trabecular bone. Further *in vivo* test using a critical-sized defect is thus warranted for future clinical application.

Conclusions

In summary, we have designed a new composite, HAp-Gemosilamine, to combine the favorable aspects of HAp-Gel, enTMOS, and PD into a single useful composite with possible tissue scaffolding applications. When being processed under low temperature (e.g., – 20 °C) before being allowed to warm up (e.g., 20 °C) for the final setting, HAp-Gemosilamine showed improved mechanical properties than those of HAp-Gemosil. In contrast, processing HAp-Gemosilamine in a manner similar to the original HAp-Gemosil (e.g., entirely at 20 °C) only resulted in poor mechanical properties. Such a strong temperature dependence behavior can be ascribed to the different temperature related polymerization kinetics for the PD formation and the enTMOS sol-gel reaction, as demonstrated by control experiments with only one component (i.e., enTMOS or dopamine) individually. For example, samples which were processed at – 20 °C showed little to no appreciable enTMOS reaction over several hours. During the same period of time, however, PD reactions were able to proceed relatively unhindered. This allowed a more extensive PD network to form before the practical multi-component based composite was warmed up for the final setting.

Deeper investigation into the morphology and phases of HAp-Gemosilamine processed under the "cold" condition revealed thoroughly mixed, but distinctly different phases within this newly designed composite. This ultimately leads to an extensively entangled polymer network incorporating HAp-Gel, believed to be responsible for the improved mechanical properties

observed in HAp-Gemosilamine than those in HAp-Gemosil. Furthermore, it was shown that these composites also maintain the biocompatibility of HAp-Gemosil materials. This demonstrates dopamine's ability to polymerize into a useful material for biological implants without causing a negative biological response. Though more testing is necessary to determine the full potential of the new HAp-Gemosilamine as a scaffolding material, the initial results are very promising and demonstrate clear advantages to using PD as an additive in bioceramic materials and using temperature as a means for controlling subsequent polymerizations within such materials.

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Figures and Captions

Figure 1

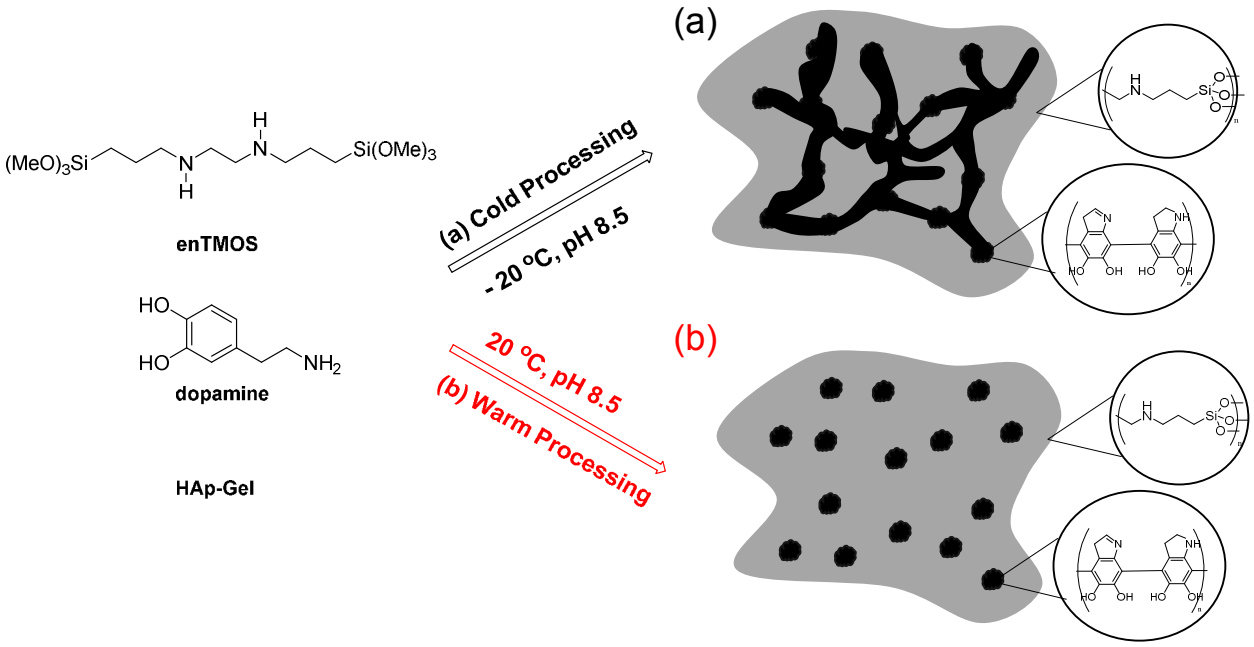


Figure 1. (a) Illustration of polymerizations involved while HAp-Gemosilamine composites are allowed to set under – 20°C. While the composite is held at a cold temperature, PD is able to effectively polymerize, while enTMOS polymerization is suppressed. This allows an extensive, interconnected PD network to form before being encapsulated by the rapid gelation of enTMOS as the composite warms to room temperature to complete setting. (b) The situation would change when HAp-Gemosilamine composites are processed at 20°C. These composites polymerize enTMOS very rapidly, leading to a highly rigid network forming rapidly. This rigid network prevents PD from forming an extensive network, and ultimately creates weak spots in the material by localizing dopamine monomers and macromolecules into small isolated pockets.

Figure 2

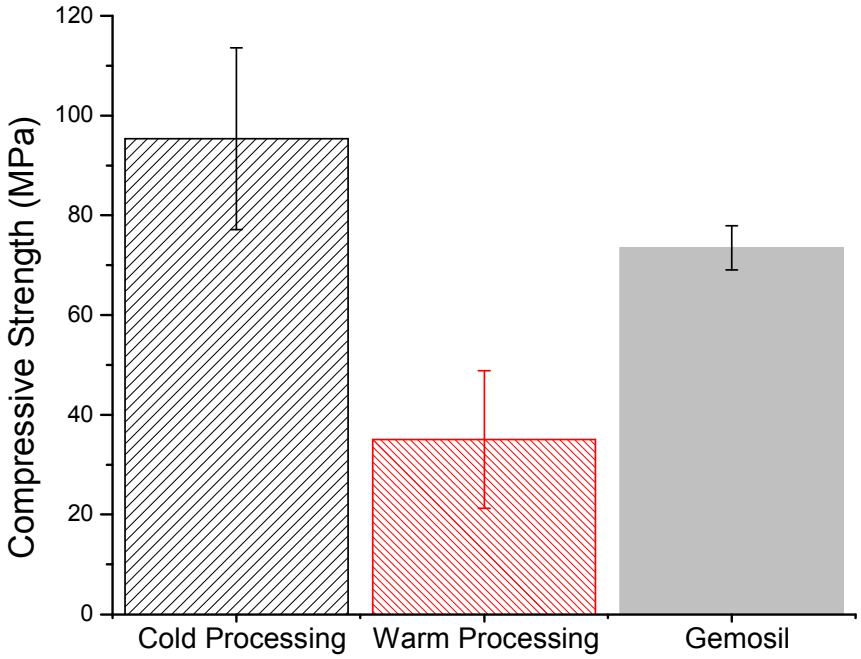


Figure 2. Compressive strength for HAp-Gemosilamine composites processed under "warm" and "cold" conditions. Additionally, data for HAp-Gemosil composites are also included for comparison.

Figure 3

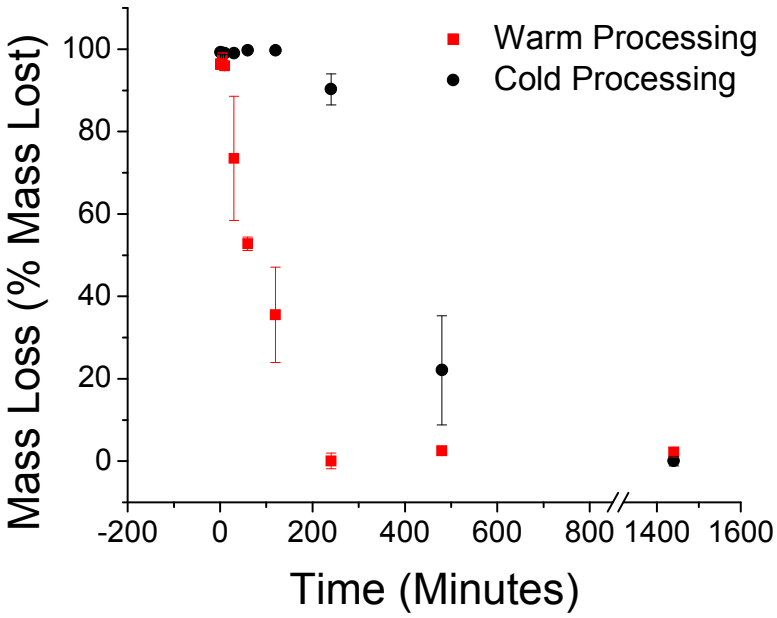


Figure 3. Mass loss data for enTMOS polymerizations. Samples which have reacted extensively will show very high mass loss, since these samples are highly cross-linked and their polymers are insoluble. This phenomenon is observed for the enTMOS samples processed at 20 °C. It can be clearly seen that the opposite trend exists for enTMOS processed at – 20 °C, where very little material has reacted even after 3 hours.

Figure 4

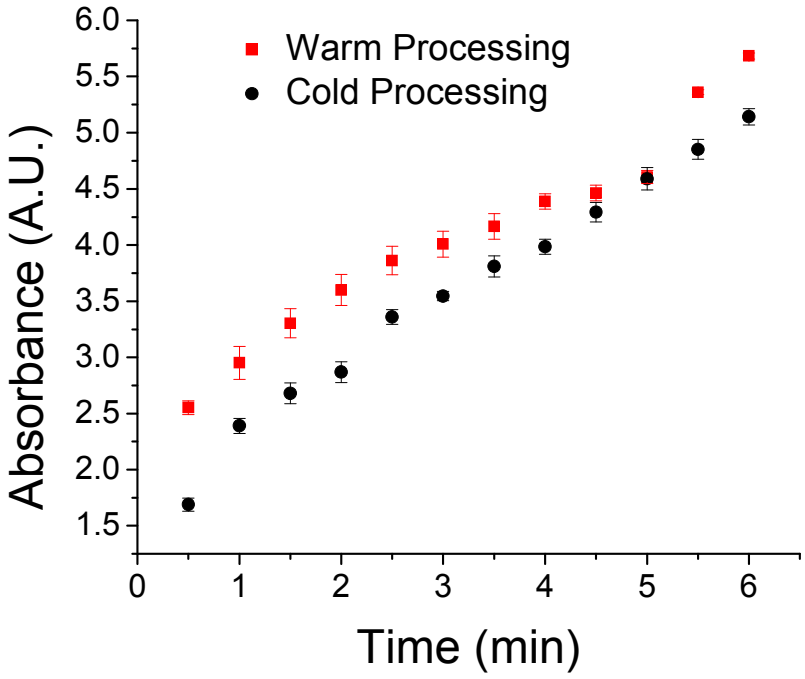


Figure 4. Reaction progress of dopamine polymerization under oxidative conditions over time. Dopamine was polymerized at both 20 °C and – 20 °C. Over this temperature range, it can be seen clearly that the polymerization of dopamine is relatively unaffected.

Figure 5

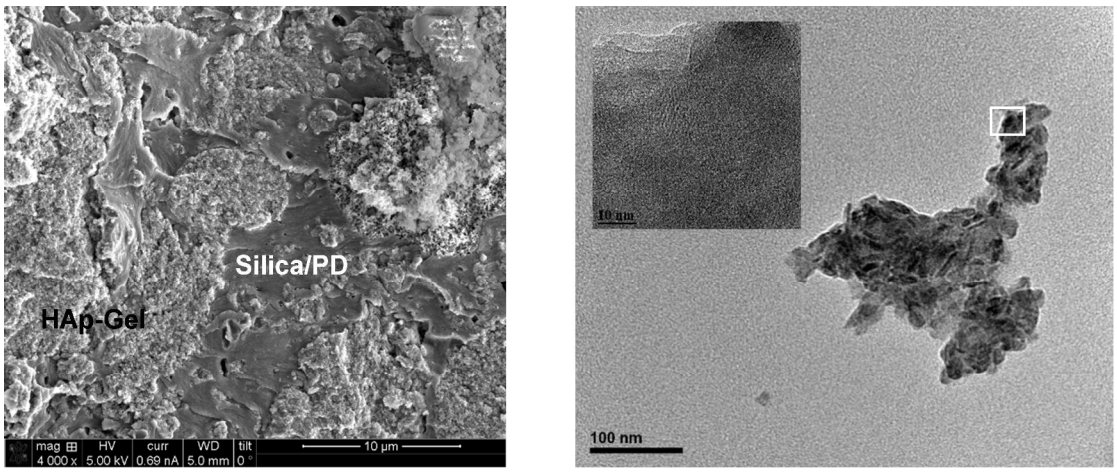


Figure 5. Left: SEM images of HAp-Gemosilamine, formed through the "cold processing", show two distinct phases of material within HAp-Gemosilamine composites. One is enriched with HAp-Gel, while the other is enriched with silica (polymer from enTMOS) and PD polymer. The two phases were statistically homogenously dispersed throughout the composite. Right: TEM images of HAp-Gemosilamine show the platelet structures (black), which represent the typical HAp-Gel nanocrystals derived from the precipitation process. The high resolution image (insert) confirms the integrity of HAp-Gel nanocrystals, as the single crystals (repeated grid pattern) are embedded inside the matrix without damage.

Figure 6

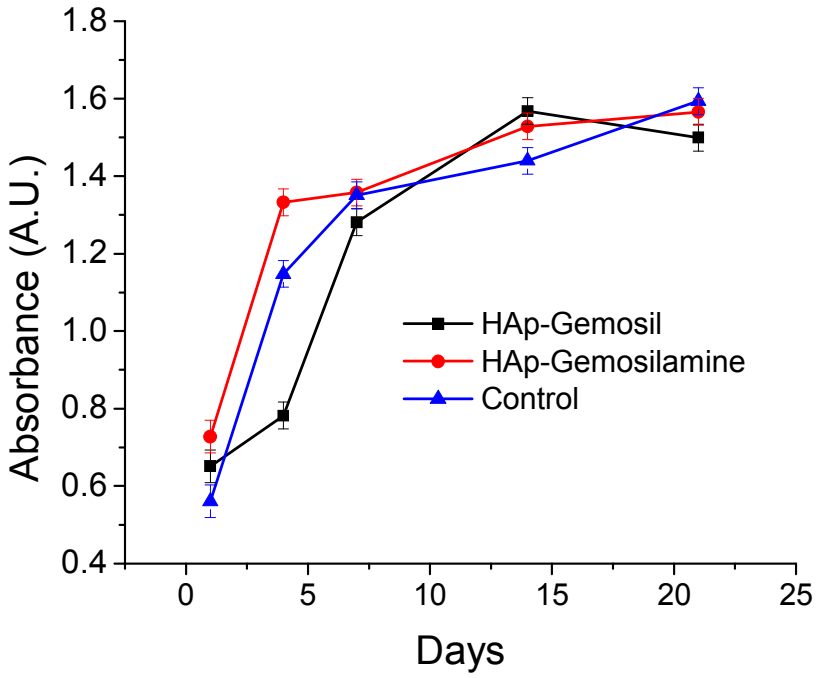


Figure 6. Cell viability data, measured by MTS assay, of MC3T3-E1 pre-osteoblasts on HAp-Gemosilamine, HAp-Gemosil, and control samples. It can be seen that all 3 substrates provide a similarly suitable substrate for cellular growth.

Figure 7

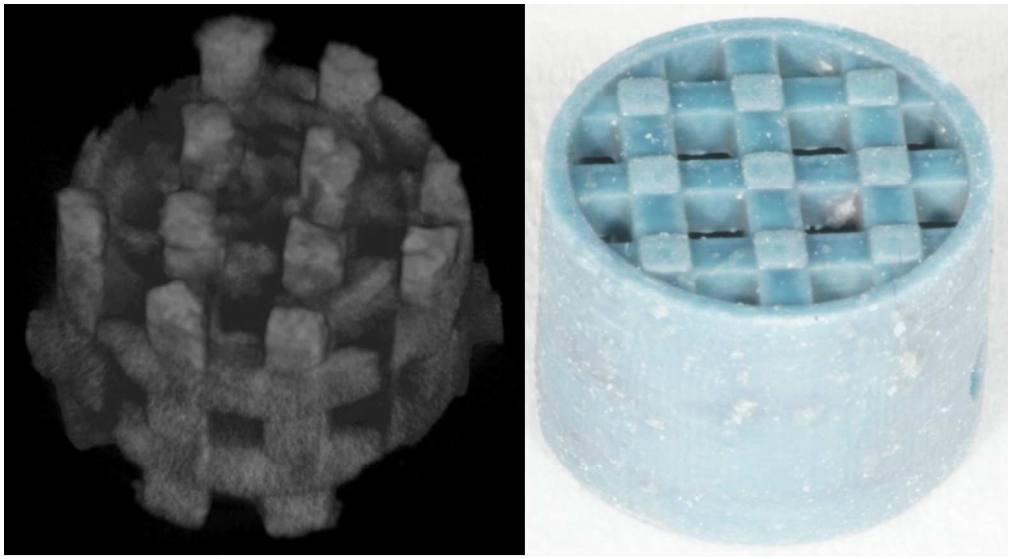
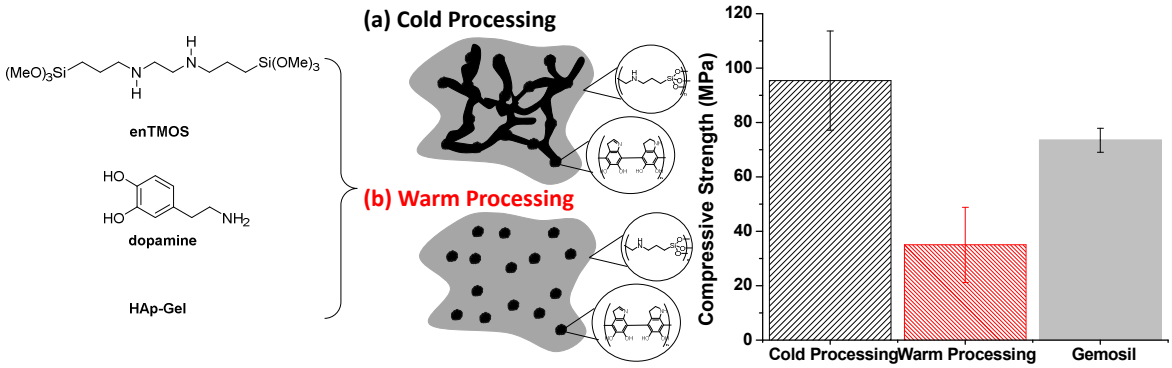


Figure 7. Computed Tomography (CT) image of the 3D scaffold (left) is made of 3D printing and an indirect scaffolding technique. Briefly, a 3D printing wax mold (right) was used to cast the HAp-Gemosilamine, from which the wax was leached and the 3D HAp-Gemosilamine scaffold was designed.

TOC Figure



processing temperature has a big impact on the mechanical property of polydopamine incorporated HAp-Gemosil