



p-Phthaloyl Chloride-Grafted SF Induced Biomineralization of Hydroxyapatites†

CHAO LIU¹, GUANGMEI CHEN^{1,*} and RONG LI²

¹Material and Chemical Engineering Department, Anhui Institute of Architecture and Industry, Hefei 230601, Anhui Province, P.R. China

²School of Chemistry and Chemical Engineering, Anhui University, Hefei 230039, Anhui Province, P.R. China

*Corresponding author: E-mail: chen_gm168@yahoo.com.cn

AJC-13250

In this paper, *p*-phthaloyl chloride (DB)-grafted SF films was employed to regulate the mineralization of hydroxyapatite in 1.5 × SBF (1.5 times of simulated body fluid) for 24 h at about 36.5 °C. It was discussed that the content of DB influence on the structure and morphology of the apatite composites deposited on the SF films. The structure and morphology of the composite materials were investigated by Fourier transform infrared, inductively coupled plasma emission spectrometer, X-ray diffraction and scanning electron microscopy. The results showed that the inorganic phase of component mainly were hydroxyapatite and they were self-assembled on the organic matrix and a three-dimensional framework was formed on the SF films. Ca/P molar ratio in the surface of biocomposites could reached 1.66. And the apatite was similarity to hydroxyapatite in natural bone in both microstructure and composition.

Key Words: Biocomposites, Hydroxyapatite, Silk fibroin.

INTRODUCTION

Recently, hydroxyapatite (HA) [mf. Ca₁₀(PO₄)₆(OH)₂] has been frequently used as biomaterials for the repair of bone tissue. It has been received much attention because of its generally excellent biocompatibility, osteoconductivity and bioactivity¹ and the ability of promoting cell-material interaction². Unfortunately, the facts of the inherent poor fracture strength and toughness of hydroxyapatite, so its clinical application was limited as a loading-bearing implant^{3,4}. In the past decades, many efforts have been focused on the development of hydroxyapatite/organic hybrid composite materials to improve its mechanical properties through biomineralization method for better used in bone tissue transplantation. These hybrid composite materials must have good biocompatibility and degradation, such as silk⁵; collagen⁶ and so on. Silk fibroin has been recently recognized as an attractive template for the biomimetic synthesis of HA/SF composite due to a unique combination of biocompatibility, biodegradability and excellent mechanical properties^{7,8}. Our previous study has described biomineralization approach to synthetic HA/SF-PASP composite, in which needle-like apatite and a three-dimensional framework is formed on the SF films⁹. On the other hand, it has been reported that carboxyl (-COOH) groups play an effective role in heterogeneous nucleation of apatite in the body environment¹⁰. Fibroin surface contains a large number of

active groups, such as amino, carboxyl, hydroxyl, *etc.* Therefore, under certain conditions, it can react with polyalcohol, polycarboxylic acid, acid anhydride, ester aldehydes, poly-functional epoxy compound.

In this work, the SF film was modified with *p*-phthaloyl chloride (DB) as to introduce carboxyl (-COOH) groups and hydroxyapatite was synthesized via biomineralization method in the employing DB-grafted SF^{11,12} as an organic matrix. Then the DB-SF films with different quantities of DB were soaked in 1.5 × SBF for 24 h at about 36.5 °C to synthetic DB-SF/HA composite after premineralization. According to our previous work, the premineralization temperature played an important role on the structure and morphology of hydroxyapatite. So in this paper, different premineralization temperatures were used to prepare samples. The structure and composite of apatite deposited on the films were investigated by FTIR, XRD, SEM and ICP. From the above results that the component of apatite deposited on the SF films mainly was low crystallinity of hydroxyapatite and three-dimensional framework is formed on the SF films.

EXPERIMENTAL

Na₂CO₃, LiBr, NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, HCl, CaCl₂, Na₂SO₄, NaOH, C₈H₄Cl₂O₂ (DB), *tris*-(hydroxymethyl)aminomethane(*tris*) and ethanol used here were analytical grade and purchased from Huipu Chemical Agents Co. Ltd. (Hangzhou China). *B. mori* silk-

†Presented to the 6th China-Korea International Conference on Multi-functional Materials and Application, 22-24 November 2012, Daejeon, Korea

worm cocoons were provided by Anhui Agricultural Research Institute.

The solution of SF was prepared according to previous method⁹. The SF films were treated with 65 % (v/v) ethanol aqueous solution for 20 min. *p*-phthaloyl chloride (DB) was dissolved in acetone, then the SF films were cast on beaker with the weight ratio of: 0.0, 0.05, 0.10, 0.15 and 0.20 (DB/SF, w/w) in 25 mL acetone solution, labeled as DB00SF, DB05SF, DB10SF, DB15SF and DB20SF, respectively. It was sealed tightly 1d and at room temperature after SF films were taken out and rinsed with acetone to remove dissolvable impurities and finally distilled water to change acryl chloride group into the carboxyl.

The SF and DB-SF were placed in 50 mL of 0.2 M CaCl₂ solution at different temperatures. After reacted for 1 min, it was rinsed with distilled water for one time and dried at room temperature, following transferred to 50 mL of 0.12 M K₂HPO₄ solution at the same temperature as in CaCl₂ solution, soaked for 1 min and washed one time with distilled water. The soaking cycles exchange of 3 times. All specimens were soaked in the 1.5 × SBF at about 36.5 °C for 24 h. After 1d the DB-SF films were taken out and rinsed with deionized water and air-drying at room temperature.

The FT-IR spectra of samples were measured with FTIR spectroscopy (Nicolet NEXUS-870, USA), the samples were mixed with KBr in the mass ratio of 1:20-30. Infrared spectra were taken in the range of 4000-400 cm⁻¹ with 2 cm⁻¹ resolution. To investigate the structure and crystallinity, the milled sample powder were analyzed on an X-ray powder diffractometer (Philips X'Pert-Pros, Dutch) using a diffractometer equipped for CuK_α radiation ($\lambda = 0.15418$ nm) in the 2 θ range of 10-50° at 40 kV and 30 mA. The morphology of apatite deposited SF films sputtered with gold was examined using scanning electron microscopy (S-4800, Hitachi, Japan) with an accelerating voltage of 5 kV. The content of Ca and P elements in the composites was analyzed by ICP (IRIS Intrepid II ICP-OES, Thermo Electron Corporation, USA). The milled composites samples were dissolved with HNO₃ and HClO₄ and then they were filled to constant volume.

RESULTS AND DISCUSSION

FTIR: Fig. 1 shows the absorption spectra of some samples. In Fig. 1, curve 'a' shows the characteristic peaks of SF through methanol treatment, the absorption bands at 1630, 1521 and 1243 cm⁻¹ are attributed to β -sheet structure of silk¹³. After soaking in 1.5 × SBF for 24 h at about 36.5 °C, the same absorption bands not only are observed, but also new absorption bands at 1033, 602 and 562 cm⁻¹, which belong to phosphate groups just as shown in curve d, appear in curves 'b' and 'c'. These results show that the SF still in the form of β -sheet structure exist after soaking in 1.5 × SBF for 24 h at about 36.5 °C. However, the positions of the amide peaks are different, the adsorption bands at amide I and II shift from 1630 to 1641 cm⁻¹ and 1521 to 1530 cm⁻¹ in curves 'b' and 'c', respectively, amide III almost unchanged. The mainly reason of red-shifted is that amide I, II and Ca²⁺ occurred chemical interaction and damaged the amide bond, so the amide I and II of IR characteristic spectrum move to higher wavenumber¹⁴. On the other hand, the absorption peaks of the phosphate groups are

similar to the hydroxyapatite. This demonstrated that the predominant component of the inorganic phase deposited on the SF films mainly was hydroxyapatite. However, the intensity of the phosphate groups of DB20SF/HA becomes stronger than that of DB00SF/HA, which indicated that the number of carbonyl groups on the surface of the silk films induced the nucleation of hydroxyapatite crystallites. Based above data analysis, we inferred that the modified film more contribute to deposition of hydroxyapatite.

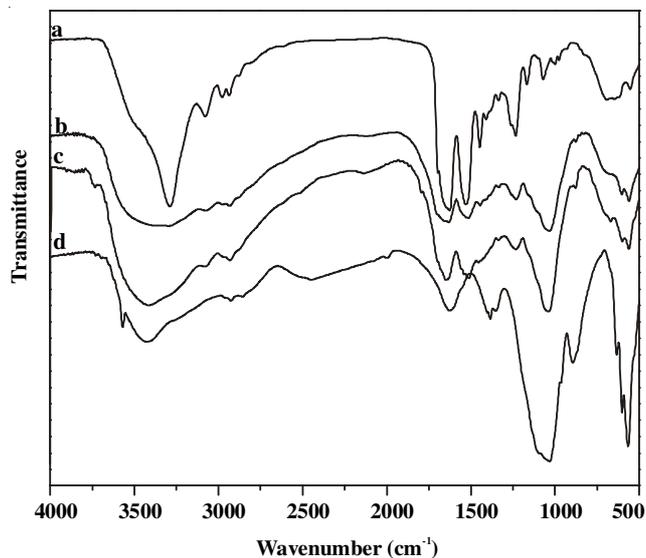


Fig. 1. FT-IR spectra of (a) SF, (b) DB00SF/HA and (c) DB20SF/HA, the deposition of the pure SF in the 1.5*SBF at 36.5 °C for 24 h, (d) HA

Because the premineralization temperatures affect the structure and morphology of hydroxyapatite on the biocomposites surface. Fig. 2 gives FTIR spectra of DB15SF/HA series at different premineralization temperature as a demonstration. With the increasing of the temperatures, the absorption band at 1033 cm⁻¹ related to phosphate groups of hydroxyapatite enhance. Interaction of Ca²⁺ with the template needs enough binding energy. The higher temperature is benefit to form of the interaction.

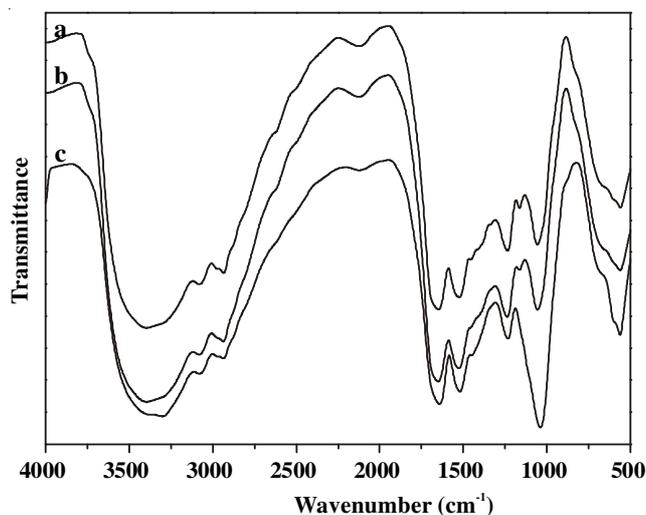


Fig. 2. FTIR spectra for DB15SF/HA samples obtained at different premineralization temperatures: a) 10 °C, b) 25 °C, c) 36.5 °C

XRD: Fig. 3 displayed XRD patterns of the samples DB00F/HA, DB05F/HA and DB20F/HA. As shown in Fig. 3, after soaking in the $1.5 \times$ SBF for 24 h at about 36.5°C , the diffraction peaks at 20.2° and 24.4° belong to SF of β -sheet structure⁸ and the clear but weak diffraction peaks are detected at 25.9° , 28.3° , 31.9° , 39.9° and 45.5° corresponding to the (002), (210), (211), (310) and (222) planes of hydroxyapatite crystal⁸, respectively. The results show that the silk fibroin and apatite coexist in composite materials and apatite deposited on the surface of the samples mainly is hydroxyapatite. Moreover, the peaks of the inorganic phase become wider and several peaks overlapped together, which indicates that the crystalline of inorganic phase is very low and crystallite size is tiny, closely similarity with the natural bone tissue¹⁵. The XRD patterns also show that the intensity increase of (002), (210), (222) planes is obviously with the increase of the content DB. It can be deduced that the more the number of carboxyl in SF film, the more favourable growth of hydroxyapatite on the DB-SF matrix.

SEM: Fig. 4 shows the morphology of the surfaces of the pure SF and DB-SF/HA with different quantities of DB. Only gold particles are observed on the surface of pure SF films (Fig. 4a), after soaking in $1.5 \times$ SBF for 24 h at about 36.5°C , spherical particles and uniform particle size of apatite are observed (Fig. 4b). From Fig. 4c and 4d it can be seen that apatite particles become regularly and completely cover the original surface of DB05SF and DB10SF films. The morphology of apatite is very different from DB10SF to DB15SF, spherical disappearing and the appearance of three-dimensional network

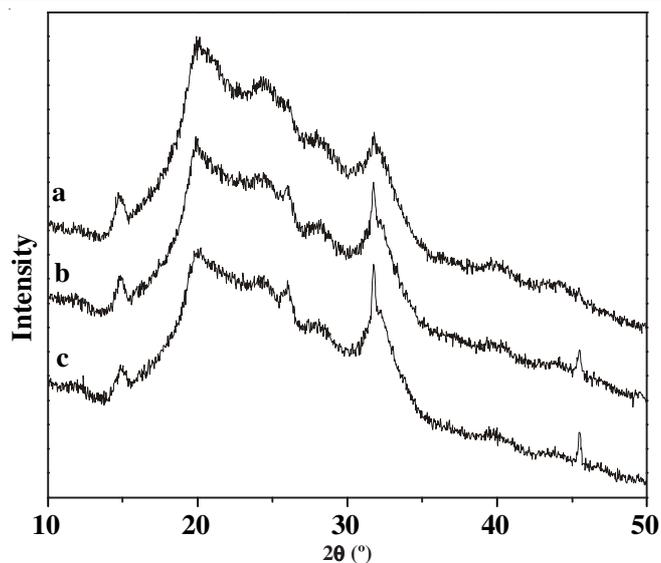


Fig. 3. X-ray diffraction of a) DB00F/HA, b) DB05F/HA and c) DB20F/HA

with many pores of irregular sizes. Finally, many pores of relatively regular sizes on the surface of the films are observed (Fig. 4f). The process of surface morphology transformation in Fig. (b-f) indicates that the number of the carboxyl group can significantly promote apatite deposited on the fibroin films. Thus, hydroxyapatite crystallites randomly aggregate into clusters and loose microstructure with irregular pore size distribution in Fig. 4b due to the pure silk fibroin film in the form of β -sheet structure containing a small amount of exposed

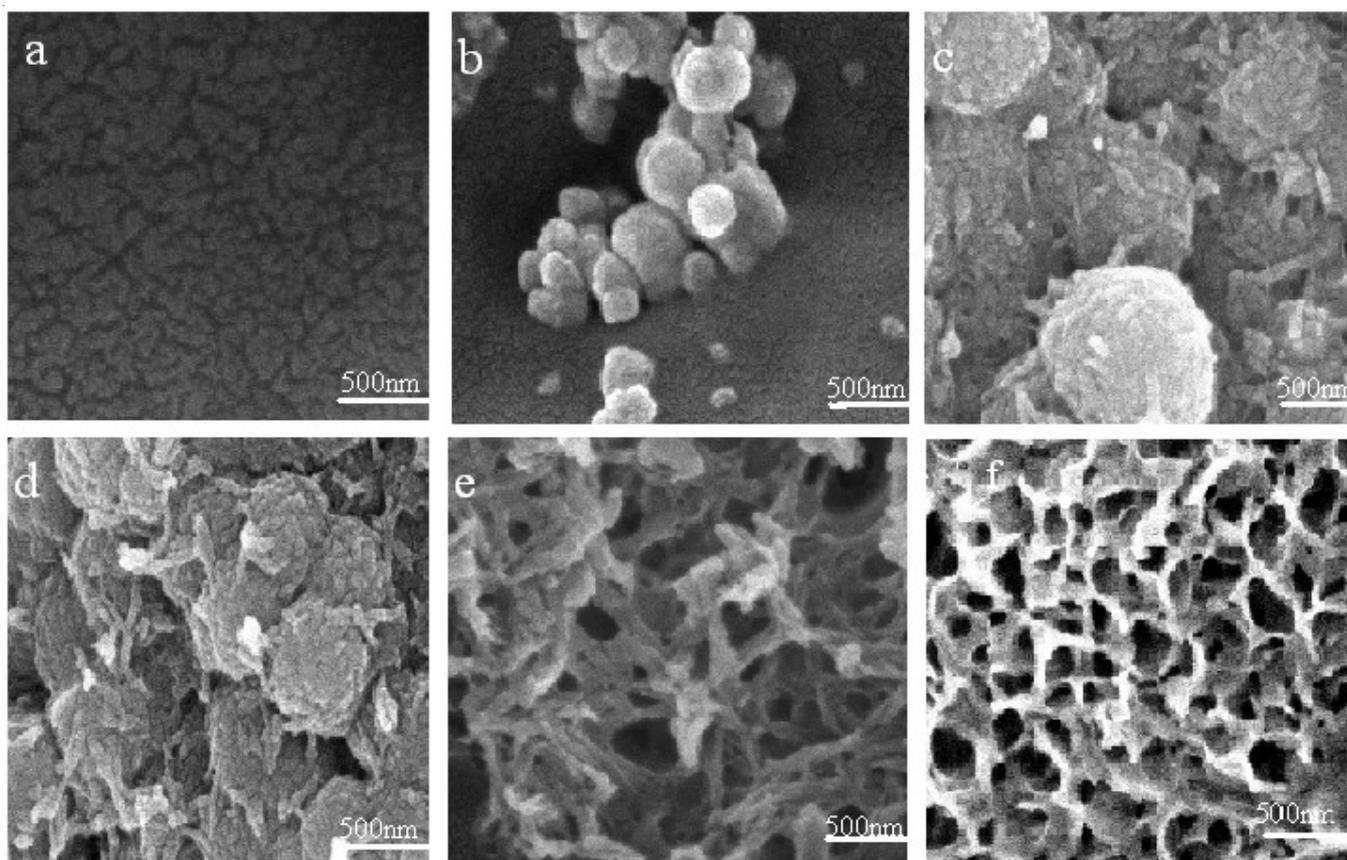


Fig. 4. SEM images of: a) DB00SF, b) DB00SF/HA, c) DB05SF/HA, d) DB10SF/HA, e) DB15SF/HA and f) DB20SF/HA. Premineralization temperature is 10°C .

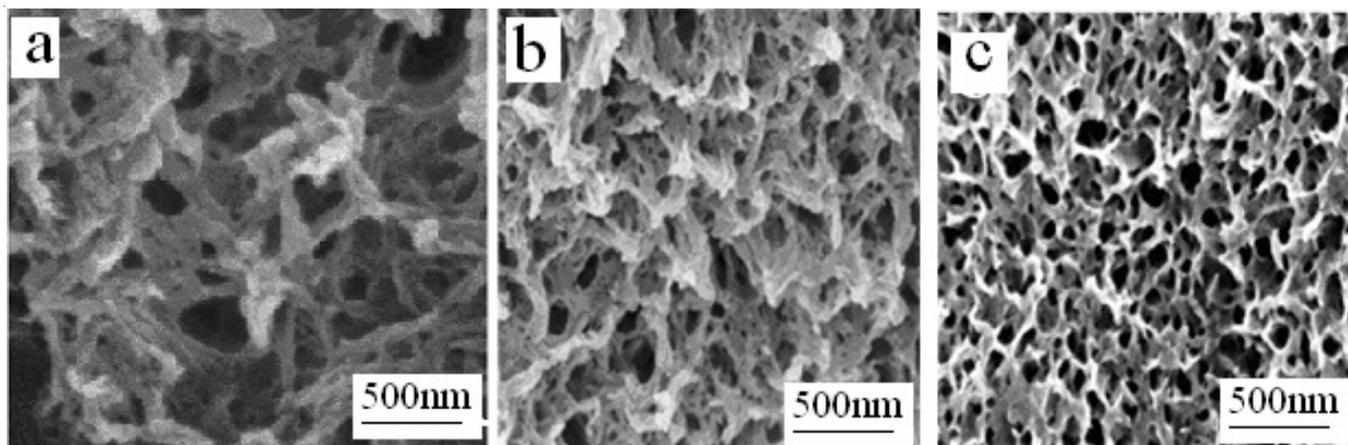


Fig. 5. SEM images of DB15SF/HA series samples premineralized at different temperatures. a) 10 °C; b) 25 °C; c) 37 °C

carboxyl on the surface of SF films, which can promote heterogeneous nucleation capability and the self-assembly of apatite crystallites on SF films. While the silk films modified with DB introducing carboxyl groups on the surface of SF films, the apatite clusters cross-linked with SF fibrils in a more intimate mode forming self-assembly of well-dispersed and three-dimensional network hydroxyapatite crystals.

Fig. 5 displays the SEM images of DB15SF/HA series samples at different premineralization temperatures. With increasing of the premineralization temperature, the apatite clusters display more compactly. The result is consistent with that of FTIR.

ICP: The ICP results of the hydroxyapatite deposition on the composites are shown in Table-1. It can be seen that the Ca/P ratio in the scaffolds increase gradually along with the addition of modification. The Ca/P ratio of the apatite deposition on the pure SF films was only 1.38, as the silk films modified with DB, the Ca/P ratio of the composites increased clearly, with the proportion of DB in the SF films increased, finally it reach 1.66, which was close to the theoretical value 1.67 of hydroxyapatite. These results proved that the biomimetic environment and the amounts of carboxyl groups in the SF films play a cooperation role with each other in the hydroxyapatite deposition. From Table-1, the premineralization temperature affects the Ca/P ratio of the composites obviously. For DB15SF/HA series, the temperature increases while the Ca/P ratio increases and is close to the theoretical value 1.67 of hydroxyapatite.

Conclusion

In this study, it is appropriate to conclude that the carboxyl groups of silk protein have an important role in regulating the mineralization of hydroxyapatite nanocrystals. SEM studies show that the morphology of the DB-SF/HA composite from the spherical particles and uniform transformation to three-dimensional network is related with the content of DB. ICP results show that the Ca/P ratio of apatite deposited on the surface of DB-SF matrix was close to the theoretical value 1.67 of hydroxyapatite. Taken together with all the results, we consider that the DB-SF/HA composite was similarity to natural bone of hydroxyapatite in both microstructure and composition. And the premineralization temperature affects the structure and morphology of the biocomposites obviously.

REFERENCES

1. S.V. Dorozhkin and M. Epple, *Angew. Chem. Int. Ed.*, **41**, 3130 (2002).
2. L. Wang, G.L. Ning and M. Senna, *Colloids Surf. A*, **254**, 159 (2005).
3. M.C. Andrew, J.V.S. Nick, G. Tom, K. David, B. Caroline, I.R. Helmtrud, R.O.C. Oreffo, V.A. Sonja, B. Teodora, S. John and M. Stephen, *Adv. Mater.*, **21**, 75 (2009).
4. K. Masanori, I. Soichiro, I. Shizuko, S. Kenichi and T. Junzo, *Biomaterials*, **22**, 1705 (2001).
5. Y. Wang, D.D. Rudym, A. Walsh, L. Abrahamsen, H.J. Kim, H.S. Kim, K.H. Carl and L.K. David, *Biomaterials*, **29**, 3415 (2008).
6. K. Nam, T. Kimura, S. Funamoto and A. Kishida, *Acta Biomater.*, **6**, 403 (2010).
7. L. Wang and C.Z. Li, *Carbohydr. Polym.*, **68**, 740 (2007).
8. C.L. Du, J. Jin, Y.C. Li, X.D. Kong, K. Wei and J. Yao, *Mater. Sci. Engg. C*, **29**, 62 (2009).
9. X.L. Ma, R. Li, L. Ru, G.W. Xu, Y.P. Huang, *eXPRESS Polym. Lett.*, **4**, 321 (2010).
10. M. Tanahashi and T. Matsuda, *J. Biomed. Mater. Res.*, **34**, 305 (1997).
11. M. Tsukada, T. Arai and S. Winkler, *J. Appl. Polym. Sci.*, **78**, 382 (2000).
12. M. Tsukada, Y. Goto, G. Freddi and H. Shiozaki, *J. Appl. Polym. Sci.*, **45**, 1189 (1992).
13. G. Freddi, P. Monti, M. Nagura, Y. Gotoh and M. Tsukada, *J. Polym. Sci. Part B: Polym. Phys.*, **35**, 841 (1997).
14. S.H. Rhee, J.D. Lee and J. Tanaka, *J. Am. Ceram. Soc.*, **83**, 2890 (2000).
15. U.J. Kim, J.Y. Park, H.J. Kim, M. Wada and D.L. Kaplan, *Biomaterials*, **26**, 2775 (2005).

TABLE-1
ICP RESULTS FROM THE DB-SF/HA COMPOSITES

Samples	Element contents in the samples		
	Ca (mg)	P (mg)	Ca/P
DB00SF/HA	14.43	8.08	1.38
DB05SF/HA	15.31	7.64	1.55
DB10SF/HA	18.37	9.06	1.57
DB15SF/HA	20.88	9.88	1.63
DB20SF/HA	28.88	18.10	1.66
DB15SF/HA-25 ^a	25.38	11.92	1.65
DB15SF/HA-36.5 ^b	32.32	15.10	1.66

^{a,b}25 and 36.5 are the premineralization temperatures