Fabrication and Micropatterning of a Hybrid Composite of Amorphous Calcium Carbonate and Poly(ethylenimine)

Hyun Sook Lee, Tai Hwan Ha,† Hyun Min Kim, and Kwan Kim*

*Department of Chemistry, Seoul National University, Seoul 151-742, Korea. *E-mail: kwankim@snu.ac.kr*
†BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea
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Amorphous calcium carbonate (ACC) can readily be prepared using ethanol as the reaction medium and ammonium carbonate as the source of carbon dioxide. Other additives, or any elaborate pH control are not needed to form the initial calcium carbonate precipitate. Ammonia generated from ammonium carbonate maintains the reaction medium in a neutral or weakly basic condition, retarding the crystallization of ACC, while ethanol itself inhibits the dissolution of ACC. The ACC prepared in this way provides a rare opportunity to fabricate molded biomimetic crystals *in vitro*, but the ACC is too fragile to be fabricated into proper shapes. The malleability of ACC is, however, greatly enhanced by incorporating poly(ethylenimine) (PEI). The ACC/PEI composite can then be fabricated, using a proper mold or template, into mechanically durable biomimetic crystals of definite shape. The ACC in the ACC/PEI composite can further be transformed into vaterite by heating under N_2 atmosphere, while the native ACC simply converts into calcite.

Key Words : Amorphous calcium carbonate, Biomimetic crystal, Poly(ethylenimine), Biological hybrid composite, Micropatterning

Introduction

Natural organisms that produce biological composites exert exquisite control over the minerals they deposit, creating materials of myriad shapes and sizes that are often of high strength.[1] Mineralized tissues are often found to contain polymorphs and individual minerals whose crystal morphology, size, and orientation are determined by local conditions and, in particular, the presence of matrix proteins or other macromolecules.[2] The processes and materials that control such crystal nucleation and growth are of great interest to materials scientists who seek to manufacture composite materials and crystalline forms analogous to those produced by nature.

Calcium carbonate is one of the most abundant biominerals and can also be grown easily under laboratory conditions. It is well known that three polymorphic forms of calcium carbonate exist, *i.e.*, vaterite, calcite, and aragonite, with each polymorph able to adopt a number of morphologies. Which polymorph is formed, and with which particular morphology, is controlled both thermodynamically and kinetically.[3] In addition to the stable polymorphs, amorphous calcium carbonate (ACC) also exists. Various organisms store their calcium and carbonate temporarily in the form of ACC.[4] This can occur because ACC is unstable and easily dissolves in water.[5] Once formed in vitro, pure ACC rapidly transforms into diverse crystalline forms. The micropatterned three-dimensional ACC phase prepared on a disordered phosphate-, methyl- or hydroxyl-terminated monolayers turns into a calcite phase.[4d] ACC synthesized by the generation of carbon dioxide from dialkyl carbonate in aqueous calcium chloride solution is transformed into vaterite by addition of a PMAA block-copolymer.[6] It has been known that polymers containing a number of ester groups in either the polymer backbone or side chains[7] or phosphorus-containing poly(ethylene glycol)[8] and poly(propylenimine) dendrimers[9] inhibit the formation of nuclei of a crystalline polymorph from the metastable ACC phase. In addition, an anionic polymer can serve as a process-directing agent which induces liquid-liquid phase separation and ultimately deposition of amorphous precursor film under a supersaturated solution of calcium carbonate.[10] Stabilized ACC also forms in nature solely for structural purposes.[11] Stabilization of ACC in that case is achieved by the cooperative action of magnesium ions and glycoproteins that are rich in glutamic and hydroxyamino acids.[4(a),4(c),12]

As the above discussion implies, biominerals in living organisms usually grow inside organic frameworks that can confine the occurrence of mineral deposition to specific sites with predetermined patterns. Hence, oriented crystals with diverse morphologies can be formed starting from nucleation at well-defined and chemically modified intracellular sites. Despite attempting to mimic living organisms, the growth of crystals with designed morphologies in vitro is still at a very primitive and immature stage, however.[13] In the orthopedic field (*i.e.*, guided bone generation), for example, natural and synthetic materials are used as gap fillers for bone defect. The filling materials are eventually reabsorbed during bone in-growth (*e.g.*, calcium phosphate and hydroxyapatite) provoked by cellular osteogenesis. Recently, calcium carbonate has been attracting as a gap filler possessing high solubility[5a,14] and low immunogenicity.[15] Calcium carbonate is superior to other biomaterials such as coral, collagen, calcium phosphate, and polytetrafluoroethylene (PTFE) in view of the in-growth rate, the immunogenicity, and the mechanical strength of grown bones.[15b,16] The use of ACC

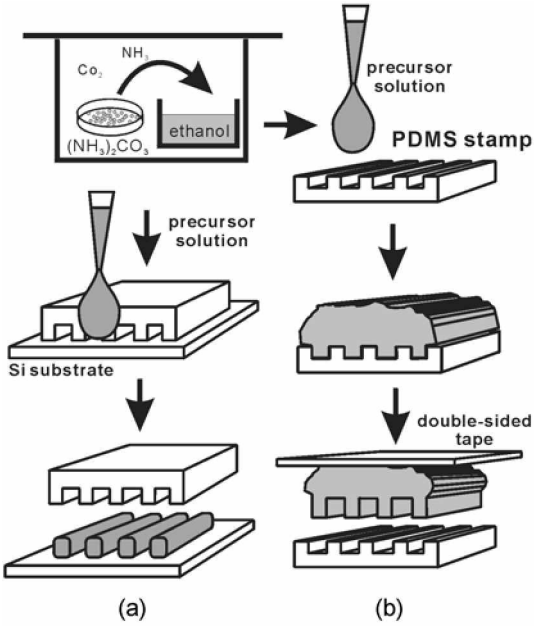
would then be desired since it is isotropic and thus can sustain mechanical strain from all directions such that it could be molded into diverse morphologies;[4d,17] ACC has also even higher solubility in physiological conditions.

Recently, we discovered that ACC could readily be prepared using ethanol as the reaction medium and ammonium carbonate as the source of carbon dioxide.[18] No other additives, nor any elaborate pH control were needed to form the initial calcium carbonate precipitate (CCP). The ammonia generated from ammonium carbonate, as well as the ethanol, was crucial, not only for the formation and stabilization of CCP, but also for its subsequent conversion to ACC by gentle heating or just keeping the CCP in ethanol. The ACC prepared in this way provided a rare opportunity to examine the feasibility of fabrication of molded biomimetic crystals resembling an implanted template usable in repairing bone defects. Unfortunately, however, the ACC was so fragile that even a two-dimensional pattern fabricated using the material was not secure. We thus devised an alternative route to synthesize stable composites composed of ACC and poly(ethylenimine) (PEI) which in fact can be assembled into robust 2-D patterns and architectures. Herein, we report a novel synthetic method to prepare those hybrid composites, and demonstrate their flexibility in forming a variety of molded calcium carbonate shapes. The transformation of ACC in the composite into more ordered crystalline phases at elevated temperatures is also demonstrated.

Experimental

The precursor of ACC was first grown in 10 mM ethanolic $CaCl_2$ solution that also contained a certain amount of PEI (average MW = 750000, 3 mg/mL), by allowing the diffusion of CO_2 vapor from $(NH_4)_2CO_3$ into the solution.[19] As the reaction proceeded, the solution phase became milky-white, and it was maintained in that way for up to 12 hrs until centrifugation; in the absence of PEI, a white precipitate was deposited at the bottom, however. The jelly-like precipitate was centrifuged at 1000 rpm, and then stored in ethanol after washing twice with the same solvent. The resulting 'fluidic calcium carbonate precipitate (CCP)' was subsequently used in micropatterning performed with an elastomeric poly(dimethylsiloxane) (PDMS) stamp. The micro molding process and the micro imprinting process adopted in this work are schematically drawn in Scheme 1.

In the micro molding process, a PDMS stamp with regular stripe patterns was laid on a silicon substrate to form micro-sized channels, and then a few hundred micro liters of as-prepared 'fluidic CCP' was dropped at the entrance site of the channel to flow inside by capillary action. In the micro imprinting process, aliquots of concentrated 'fluidic CCP' were spread on a PDMS stamp, and were left to dry for 6 hrs under ambient conditions or gently heated to 80 °C in an oven; concentrated 'CCP' was obtained by discarding the ethanol supernatant after storing the as-prepared 'CCP' in a stationary state for 2 hrs. The shaped calcium carbonate layer was stripped off using double-sided adhesive carbon tape.



Scheme 1. Schematic diagrams of micropatterning protocols; (a) micro molding process and (b) micro imprinting process. See text.

The structural analyses of the dried CCP were carried out by FT-IR spectroscopy (Bruker IFS 113v FT-IR spectrometer), X-ray diffractometry (XRD, Rigaku Model D/Max-3C) and temperature-dependent X-ray diffractometry (Bruker GADDS). More specifically, the infrared spectra of the powdered samples were obtained using diffuse reflectance optics (Harrick Model DRA-2CO).[20] The morphology of the patterned calcium carbonate was observed using a field emission scanning electron microscope (FE-SEM, JEOL JSM-6700F) and an atomic force microscope (AFM, Digital Instruments Nanoscope IIIa). Themogravimetric analysis (TGA) of CCP was conducted with a TA Instrument 2050 thermogravimetric analyzer in a N_2 atmosphere flowing at 110 mL/min, with a heating rate of 10 °C/min.

Results and Discussion

A series of different amounts of poly(ethylenimine) (PEI) were added into the reaction vessel of ethanolic $CaCl_2$ solution to obtain the calcium carbonate precipitate (CCP), as mentioned in the Experimental section. The necessary carbon dioxide was obtained from the sublimation of ammonium carbonate. The formation of CCP was seen to accelerate in the presence of PEI compared to the case without PEI. This can be understood on the grounds that PEI solution is already basic, while in its absence the reaction medium becomes basic after ammonia is diffused into the medium by the sublimation of ammonium carbonate.

The CCP was confirmed to be a composite of ACC and PEI, from XRD and FT-IR spectroscopy. As can be seen in Figure 1(a), the XRD pattern is silent and featureless, indicative of the amorphous nature of the CCP; for comparison, the XRD pattern for authentic calcite that has been grown in

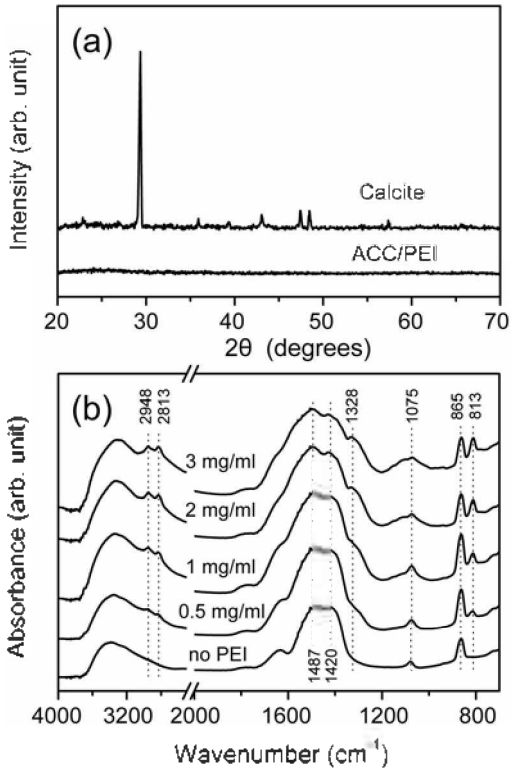


Figure 1. (a) XRD patterns of CCP and authentic calcite, (b) infrared spectra of CCP prepared at diverse concentration of PEI.

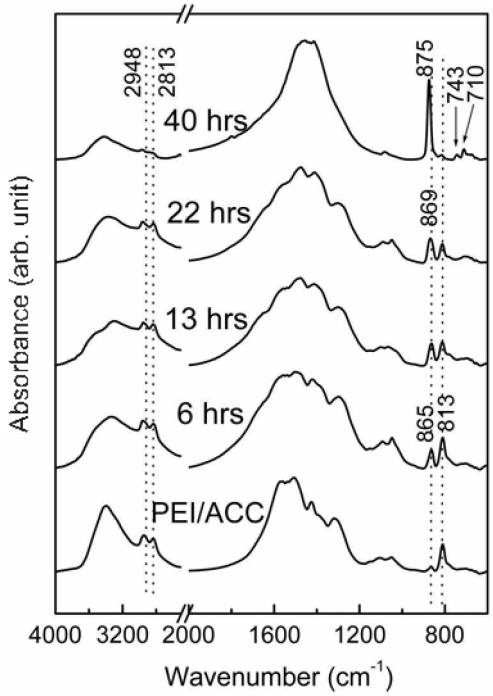


Figure 2. Infrared spectra of ACC/PEI composite taken after soaking in water for a certain period of time and then dried.

water is also shown in Figure 1(a). On the other hand, the presence of the ACC phase is characterized, in the infrared spectra shown in Figure 1(b), by the antisymmetric stretching (ν_3) bands of the carbonate ions that are split at 1420 and 1487 cm^{-1} due to the lack of environmental symmetry.[4(a),21] The band at 1075 cm^{-1} can be assigned to the symmetric stretching mode (ν_1) of the carbonate ion, and its broad features are also attributed to the absence of symmetry.[4(a),21] The carbonate out-of-plane bending (ν_2) band at 865 cm^{-1} is also broader than its counter band in the crystalline phase (data not shown). Incorporation of PEI is clear from the presence of the CH_2 stretching bands at 2948 and 2813 cm^{-1}, which become intensified as the content of PEI increases. In addition, a shoulder peak at 1328 cm^{-1}, as well as a broad peak at ~1100 cm^{-1}, is also attributed to PEI.[22]

In Figure 1(b), there is an intriguing band at 813 cm^{-1} which becomes more intense as the amount of PEI increases. In our previous work, a similar band was identified, albeit weak, at ~820 cm^{-1}, and the band was attributed to out-of-plane bending of the carbonate that was under interaction with ammonia.[18] Upon heating gently to ~100 °C to obtain pure ACC from the CCP, the band completely disappeared, however, owing to the vaporization of the ammonia (*vide infra*). The band observed herein at 813 cm^{-1} for the ACC/PEI composite can also be attributed to out-of-plane bending of the carbonate that is now under interaction with the amine group of PEI.

Conventional ACC is known to transform rapidly into the more stable crystalline polymorph of calcium carbonate.[4c,7] We have reported that the ammonia-stabilized ACC converts to calcite in a week at ambient conditions of 40-60% relative humidity.[18] The ACC/PEI composite prepared in this work is hardly subjected to transformation into calcite, at least not for 2 months. The enhanced stability is attributed to the polymeric nature of PEI with extremely low vapor pressure compared to the ammonia incorporated in simple CCPs (*vide supra*). The presence of amine groups in the coordination sphere around the calcium and/or carbonate ions is supposed to prevent its reorganization into stable crystalline phases. When the ACC/PEI composite is soaked in water, PEI gradually dissolves out, however, allowing the formation of crystalline calcium carbonates. As shown in Figure 2, the CH_2 stretching bands at 2800-2900 cm^{-1} (due to the PEI backbone) and the out-of-plane bending band of carbonate at 813 cm^{-1} (under interaction with amine groups) are weakened, while the carbonate bending band at 875 cm^{-1}, indicative of the formation of crystalline phases, grows upon soaking in water; after soaking in water for 40 hrs, the characteristic infrared peaks of vaterite and calcite appear distinctly at 743 and 710 cm^{-1}, respectively.

The polymeric network of PEI also affects the thermal characteristics of ACC. As shown in Figure 3(a), the simple ACC made in ethanol undergoes two drastic changes in the thermo-gravimetric analysis. The one corresponds to a continuous loss of water below 200 °C and the other corresponds to the transition from calcite to calcium oxide, along with the loss of carbon dioxide. As can be seen in a DSC data (see the inset of Figure 3(a)), the transition from ACC to calcite occurs at ~350 °C. This transformation was further confirmed by variable temperature XRD experiment, showing calcite peaks near ~340 °C (Figure 3(b)). In the case

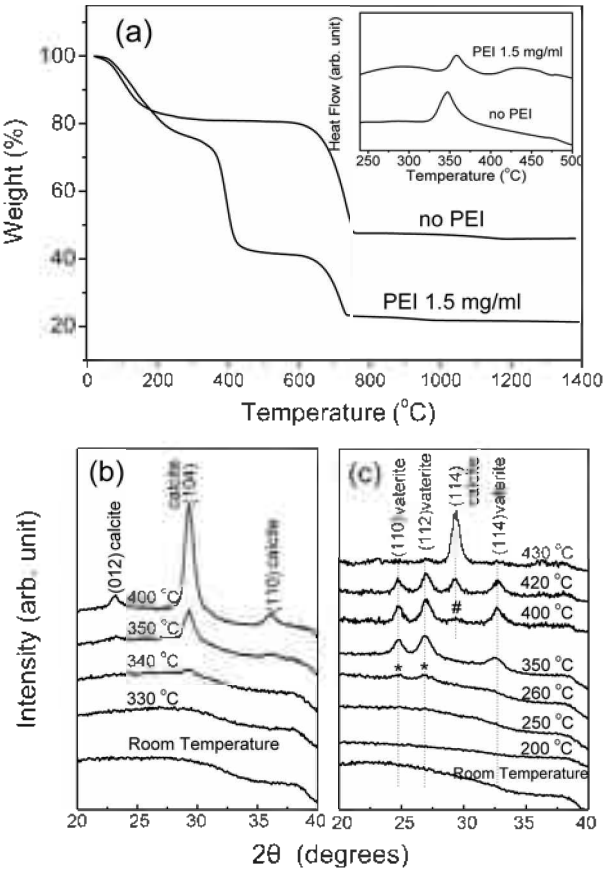


Figure 3. (a) TGA and DSC analyses of the simple ACC and the ACC/PEI composite, and temperature-dependent XRD patterns of (b) the simple ACC and (c) the ACC/PEI composite.

of ACC/PEI composite, an additional gravimetric loss occurs around 400 °C due to the thermal decomposition of the incorporated PEI. The presence of an exothermic peak at ~360 °C in the DSC data for both the simple ACC and the ACC/PEI composite suggests, however, that PEI affects little the thermal stability of the ACC phase. Interestingly, a more careful examination reveals that the presence of PEI accelerates a phase transformation of ACC. As can be seen in Figure 3(c), the temperature-dependent XRD data clearly indicates that ACC is converted to vaterite at ~260 °C, while calcites are forming at ~400 °C where PEI is subjected to decompose. In fact, vaterite is known to form when heating an ACC composite with poly(ε-caprolactone) or poly(methyl methacrylate).[7] The amine groups in PEI may similarly trigger the formation of vaterite for the ACC/PEI composite.

For a comparative purpose, a composite of ACC with poly(ethylene glycol) (PEG) or poly(acrylic acid) (PAA) was also prepared by replacing PEI with PEG (average MW = 3400, 3 mg/mL) or PAA (average MW = 750000, 3 mg/mL) in the reaction medium. Even when PEG was used instead of PEI, ACC was formed, but the ACC phase was present to be completely isolated from PEG. The thermal characteristics of the product were hardly different from those of the authentic ACC. In contrast, when PAA was used instead of PEI, a composite of ACC incorporating PAA

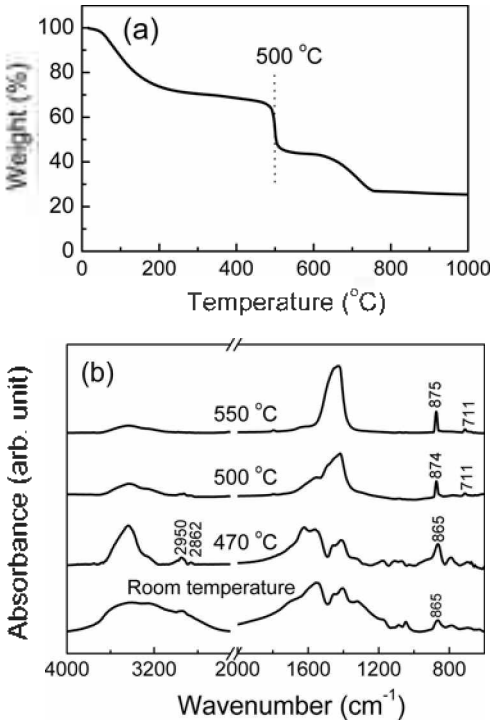


Figure 4. (a) TGA analysis and (b) infrared spectra of the ACC/PAA composite heated to 470, 500, and 550 °C.

(called ACC/PAA) was formed, exhibiting enhanced thermal stability. As shown in Figure 4(a), the ACC/PAA composite undergoes three drastic changes in the thermo-gravimetric analysis. The first gravimetric loss corresponds to a continuous loss of water below 200 °C and the second occurs around 500 °C due to the thermal decomposition of the incorporated PAA. Another corresponds to the transition from calcite to calcium oxide, along with the loss of carbon dioxide. The ACC phase coordinated with PAA sustains the amorphous characteristics up to 470 °C that can be inferred from the out-of-plane bending mode of carbonate at 865 cm^{-1} of ACC. At 500 °C, near the decomposition point of PAA, the bands of PAA at ~2900, 1670, 1320 cm^{-1} disappear, while the bands associated with calcite develop at 875 and 711cm^{-1}.

It is very intriguing that the phase transformation of ACC into vaterite is highly dependent on the kind of polymers incorporated into the composite. For pure ACC, the transition occurs at 350 °C, but PEI once incorporated accelerates the transition to occur at ~260 °C, while PAA retards the transition to occur at ~470 °C. The higher thermal stability of the ACC/PAA composite is attributed to the coordination of the carboxyl groups of PAA with the calcium ions of ACC. For the case of the ACC/PEI composite, positively charged amine groups of PEI are thought to interact more favorably with the negatively charged carboxylate groups of vaterite than ACC at elevated temperatures.[23] In a similar fashion to the usual biogenic inorganic materials, the present ACC/PEI composite may then be used in the fabrication of molded structures that will show high mechanical strength. The characteristics of the amorphous phase must be advantageous in building up biomimetic architectures since there is no

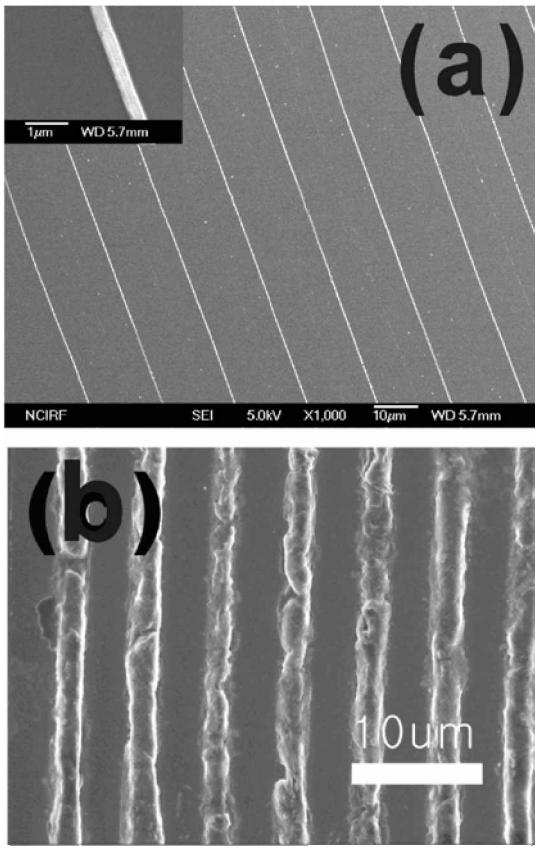


Figure 5. FE-SEM images of the micro-patterned ACC on a silicon wafer *via* micro molding method using (a) the ACC/PEI composite and (b) the simple ACC.

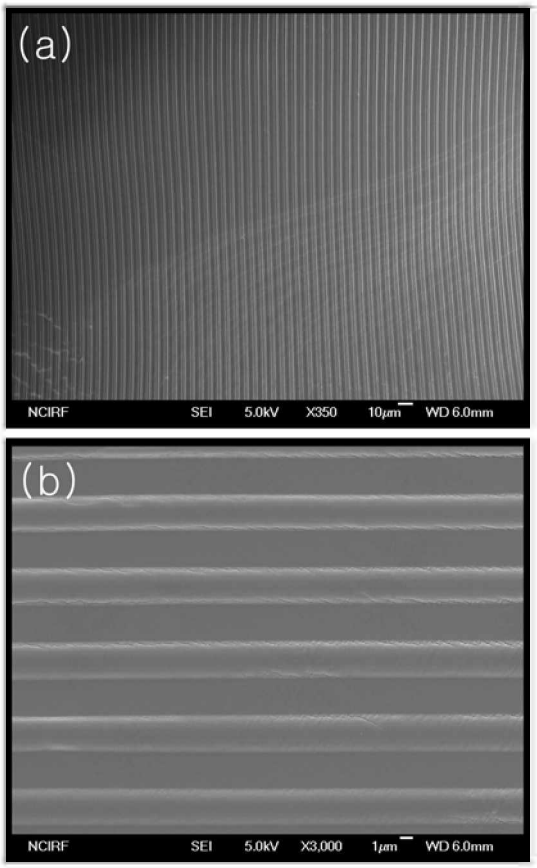


Figure 6. FE-SEM images of the ACC/PEI composite that has been fabricated *via* micro imprinting process, shown with different magnifications.

preference in orientation.

As mentioned in the Experimental section, micropatterning was conducted using two protocols,[24] one *via* a micro molding process and the other *via* a micro imprinting process (see also Scheme 1). Figure 5(a) shows the FE-SEM image of the ACC that has been patterned on a silicon substrate *via* the micro molding method; in this specific case, a drop of the as-prepared CCP solution was introduced at the entrance of the micro-channel [15 × 5 (width × height) μm^2] formed by the contact of PDMS stamp with a silicon wafer. After one hour incubation at ambient condition, the PDMS stamp was carefully removed and the remaining wafer was further kept in an anhydrous chamber for three hours. Relatively sharp lines of ~400 nm width are observed in the FE-SEM image, as shown in Figure 5(a). The height is measured to be 350-500 nm in the AFM measurement, which is far smaller than the actual depth of the PDMS stamp, *i.e.*, 3 μm, however. Supposedly, this was because the as-prepared CCP solution was too dilute to be drawn into the channel rapidly by the capillary force.[25] When micro-patterning is conducted using ethanolic CCP without PEI, very uneven and irregular patterns are produced, as shown in Figure 5(b). Local coagulation of CCP seems to take place during the evaporation of ethanol. A fairly even pattern obtainable in the presence of PEI may then be attributed to the retardation of solvent evaporation associated with the hygroscopic nature of PEI.

Figure 6 shows the FE-SEM images of ACC aggregates (with different magnification) that have been fabricated *via* the micro imprinting process. Regularly spaced and defect-free parallel line structures are seen over large areas (*i.e.*, 300 μm × 300 μm) in Figure 6(a); the image is the negative replica of the PDMS stamp employed initially. The magnified image in Figure 6(b) reveals that the surface is, in fact, very smooth and continuous; a very homogeneous pattern can be obtained even on a millimeter scale. As mentioned in the Introduction, the imprinting method cannot be applied using the CCP without PEI; as the solvent evaporates, the gel-like CCP turns into ACC in powdered state. Owing to the excessive stability of the ACC/PEI, the patterned structure in Figure 6 remains in a stable state without converting to calcite for at least 2 months.

In conclusion, it is evident that the stability and malleability of ACC are greatly enhanced upon the incorporation of PEI. When heated under N_2 atmosphere, the ACC in the ACC/PEI composite transforms into vaterite, while the native ACC simply converts into calcite. Since the precursor phase formed in ethanol is fluidic and gel-like, the composite can be easily fabricated into any shape as required by a template in clinical implants. The prospects for application of the present ACC/PEI composite are thus expected to be very high.

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