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Artificial peptides binding to the *c* face of hydroxyapatite obtained by molecular display technology†

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Artificial liner peptides that bind to the *c* face of hydroxyapatite (HA) were obtained by using the mRNA display method. The specific attachment to the *c* face was characterized with the adsorption isotherm of the peptides. The crystal growth of HA was found to be modulated by the particular peptides. The results of this study suggest that the amino acid sequence including Ala-Asn-Thr (ANT) is essential for the binding specificity.

Introduction

It is well-known that hard tissues of mammals, which are composed primarily of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HA), have high functionality derived from their complex and hierarchical structure.[1,2] The protein has been reported to be essential for the control of bone formation.[6] For instance, amelogenin, *i.e.*, the protein produced by ameloblasts in teeth, works as a matrix to guide the growth direction of HA.[3] Consequently, the enamel layer consists of a highly organized array of HA crystallites elongated along the *c* axis.[4] Osteocalcin, *i.e.*, the most abundant non-collagenous protein of the bone extracellular matrix, has been suggested to regulate the crystal growth of HA *via* specific adsorption to the *c* face.[5] Even though the interaction of proteins and HA has not been completely clarified due to the complexity of their molecular structure, the specific macromolecules finely control the nucleation and the crystal growth of HA. The proteins and their related molecules could be utilized as a regulator for use in biomaterial engineering. Thus, it is highly important to clarify the interaction between proteins and specific faces of HA in order to understand the process of biomineralizaton and develop bio-related material engineering. However, the extraction and synthesis of particular proteins for engineering applications are impractical. An osteopontin phosphopeptide binding to a specific face of calcium oxalate monohydrate was reported to modulate the crystal growth.[7] In this work, therefore, we obtained low-molecular-weight peptides that can be utilized to regulate the crystal growth of HA due to their binding specificity by using molecular display technology.

Fundamentally, the *a* and *c* faces of HA have different adsorption characteristics for various proteins because of the specific ionic alignments on the surface.[8] The calcium-rich *a* face and the phosphate-rich *c* face are assumed to be positively and negatively charged, respectively. However, the adsorption mechanism of the particular macromolecules has not been simply explained by the adsorbability of a single amino acid or their charge state. Pan *et al.* studied the adsorption properties of amino acids on the *a* and *c* faces of HA by means of a molecular simulation. Glycine and glutamic acid have been reported to attach specifically on *a* and *c* faces, respectively.[8] On the other hand, Matsumoto *et al.* suggested the similarity of the adsorbability of these molecules from the change in the solubility of HA.[9] Hirakura *et al.* reported that the charge and the size of the proteins were not critical for attachment to the specific faces of HA.[10] The essence of the interaction between the macromolecules and HA is not sufficiently understood.

Molecular display techniques have been used to identify, from a large number of different amino acid sequences, those proteins or peptides which bind specifically to targeted biomolecules.[11,12] Among the techniques, a phage display or cell surface display was applied in the field of material chemistry, and several peptides binding to metals, inorganics, and organic macromolecules were identified.[13,14] Some of the peptides binding to the specific atomic arrangement were used to fabricate nanoscale materials.[15] Gungormus *et al.* reported the amino acid sequence of HA-attaching peptides that are identified by using a phage display; however, the crystallographic face attaching to the peptides has not been specified.[16]

In the current research, we synthesized HA disks exposing a large area of the *c* face as a target by modifying the method proposed by Ohta *et al.*[17,18] The mRNA display technique was

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† Electronic supplementary information (ESI) available: Amino acid sequences of 40 peptides adsorbing to the *c* face pellets after six rounds of the *in vitro* selection, CD spectra of the modified peptides, SEM images of HA crystals grown for 168 h on seed HA grains, XRD patterns and SEM images of the surface of sintered *c*-axis oriented and unoriented HAp. See DOI: 10.1039/c2ra22382a

then applied to identify peptides attaching to the *c* face. This method is superior to other display technologies[19] in that makes it possible to identify more proteins or peptides binding to the targeted substances from a larger number of different amino acid sequences. We successfully identified artificial liner peptides that bind to the *c* face of HA. The interaction between the peptides and the specific face is discussed on the basis of their influence on the crystal growth of HA in a supersaturated solution.

Experimental section

Aggregates of needle-like HA nanostructures arranged in the *c* direction were prepared by hydrolysis of dicalcium phosphate dehydrate ($CaHPO_4 \cdot 2H_2O$) at 95 °C for 6 h in a 10 wt% NaOH aqueous solution. The resultant powder (0.15 g) was compressed into disk-like pellets. We obtained *c* face HA pellets by sintering the compacted powder at 1200 °C for 24 h (Fig. S1, ESI†). Unoriented pellets were prepared as a reference by sintering a disk consisting of commercial HA powder (Junsei Chemical 96.0%) at 1200 °C for 24 h.

Using the *c* face pellet, the mRNA display method was performed to isolate peptides that interact with the surface. A library consisting of 16-mer peptides tagged with hexahistidine at the C-terminal was displayed on mRNA molecules encoding each amino acid sequence.

The library of mRNA-peptide fusion molecules was purified by using His-tag affinity resin, reverse-transcribed, and then applied to the *c* face pellet. After washing with Tris-buffered saline (pH 7.4), the surface of the pellet was dissolved with a Glycine–HCl buffer (pH 3.0), and the fusion molecules were then recovered with the Glycine–HCl buffer. A DNA portion of the fusion molecules was amplified by polymerase chain reaction (PCR) and then used as a library for the next round of selection procedures. After six rounds of iterative selection and amplification, we identified candidate peptide sequences that can specifically bind to the *c* face of HA.

To evaluate the amounts of adsorbed proteins, fluorescein was covalently bound to the N-terminus of all the peptides. First, certain amounts of the fluorescent peptides were deposited on the HA pellets. We then obtained the standard curve for the relationship between the fluorescence intensity and the amount of fluorescent peptides on the HA pellets. The *c* face HA pellets and the unoriented HA pellets were immersed in a 300 mm^3 solution containing the fluorescent peptides at a concentration ranging from 1.25 to 250 mg dm^{-3}. After incubation for 24 h at 25 °C, the pellets were immersed in purified water to rinse the excess amount of peptides on the surface. The amount of peptides adsorbed on the pellets was estimated by means of the fluorescence intensity by using the standard curve.

For the crystal growth of HA, a solution containing 3.4 mmol dm^{-3} Ca^{2+}, 2.0 mmol dm^{-3} HPO_4^{2-}, and 10.0 mmol dm^{-3} Mg^{2+} was prepared at pH 2 by mixing $CaCl_2$ (Junsei Chemical 99.0%), K_2HPO_4 (Junsei Chemical 99.0%), $MgCl_2 \cdot 6H_2O$ (Junsei Chemical 99.0%), and $HCl_{aq.}$ (Junsei Chemical 35.0%). The pH was then adjusted to 6.4 by using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 25 °C. After 125 μg of peptide was added to 1 cm^3 of the solution, the pH was adjusted to 6.4 again by using HEPES and diluted $HCl_{aq.}$ at 25 °C. In this work, the growth rate of HA crystal was limited with the addition of Mg^{2+} and the adjustment of the pH to a weak acidity to achieve the epitaxial growth on the seed HA grains. The *c* face pellet was immersed and incubated at 25 °C for 24 h. For the continuous growth of HA for 168 h, 3.4 mm^3 of a 100 mmol dm^{-3} $CaCl_2$ solution and 2.0 mm^3 of a 100 mmol dm^{-3} K_2HPO_4 solution were added every 24 h. The resultant samples were washed with purified water and dried at 60 °C for 24 h in air. We observed the formation of oriented bundles consisting of HA needles elongated in the *c* axis on each crystal grain of the unoriented HA pellet (Fig. S2, ESI†). This suggests that epitaxial growth in the *c* direction occurs on an HA substrate under the mild supersaturated conditions.

The morphology of the products was characterized using a Hitachi S-4700 field-emission scanning electron microscope (FESEM) and a Shimadzu SPM-9600 scanning probe microscope (SPM). The X-ray diffraction (XRD) patterns were recorded using Rigaku MiniFlex II with Cu-Kα radiation. The fluorescence intensity was evaluated by a JASCO FP-6500 spectrophotofluorometer. Circular dichroism (CD) spectrometry was conducted with 125 ppm peptide solutions by using a JASCO J-500.

Results and discussion

Using the *c* face pellet, the mRNA display method was performed to isolate peptides that interact with the surface. A library consisting of 16-mer peptides tagged with hexahistidine at the C-terminal was displayed on mRNA molecules encoding each amino acid sequence. After six rounds of iterative selection and amplification, we identified candidate peptide sequences that can specifically bind to the *c* face of HA. We analyzed the amino acid sequences of 40 peptides that were randomly chosen from peptides adsorbing to the *c* face pellets after six rounds of *in vitro* selection (Table S1, ESI†). In the peptides, we observed several common partial sequences of three or four amino acids; RQT, ANTN, AANT, TED, TDP, HRT, NRT, TTAN, TKT, SPT, THH, and TKT (T: threonine, K: lysine, S: serine, P: proline, E: glutamic acid, Q: glutamine, H: histidine, R: arginine, L: leucine, D: aspartic acid, N: asparagine). Five peptides (pA–pE) containing two or more common partial sequences of amino acids are listed in Table 1.

Because fluorescein was bound to an N-terminus of the peptides, the difference in the amounts of peptides attaching to the surfaces was evaluated from the fluorescence intensity. As shown in Fig. 1, the five peptides are naturally observed on the *c* face pellet. On the other hand, a very small amount of the peptides adsorbed to the unoriented HA grains. This means

Table 1 Sequences and isoelectric points of *c* face-attaching peptides that were selected due to the presence of two or more common partial sequences of amino acids

	Sequence[a]	Isoelectric point	Saturated adsorption amount[b] (peptide/nm^2)	Adsorption constant[b] (nm^2 mg^{-1})
pA:	NPPTRQTKPKRVANTN	12.02	1.07	114
pB:	SAANTTQLNTPTEDNEP	3.57	0.90	70
pC:	TTDPHRTDNNRTKYQT	8.28	0.86	34
pD:	TDPPSPKHHCLPTTAN	6.61	1.17	23
pE:	TKTSPTPENPTQQHRT	8.44	1.05	14
pAa:	TPPNRQTKPKRVANTN[c]	12.02	1.02	94
pAb:	NPPTTQRKPKRVANTN[c]	12.02	1.04	67
pAc:	NPPTRQTVRKPKANTN[c]	12.02	0.99	85
pAd:	NPPTRQTKPKRVNTNA[c]	12.02	0.93	109
pAr:	NTNAVRKPKTQRTPPN[c]	12.02	0.69	21

[a] Each letter represents an amino acid (T: threonine, K: lysine, S: serine, P: proline, E: glutamic acid, Q: glutamine, H: histidine, R: arginine, L: leucine, D: aspartic acid, N: asparagine, Y: tyrosine). The partial sequences of amino acids which commonly appear in 40 peptides adsorbing to the *c* face pellets after six rounds of the *in vitro* selection (Table S1, ESI†) are shown with colours. [b] The saturated adsorption density and adsorption constants were estimated from adsorption isotherms at 25 °C. [c] Peptides pAa–pAd were prepared with partial modification of pA.

that the peptides identified with the mRNA display method are highly specific to the *c* face. Fig. 2a shows the adsorption isotherms of the peptides on the *c* face and unoriented surfaces. The density of peptides adsorbing on the surface was calculated by assuming that the surface is completely flat and smooth. The adsorption properties of artificial peptides, such as the adsorption constant and the saturated adsorption density, were estimated from the adsorption isotherms with the Langmuir adsorption equation (Table 1). Because the molecular size of pA was calculated to be 2.3 nm^3, the saturated adsorption density, *ca.* 1.0 molecule/nm^2, suggests that the peptide molecules completely covered the HA surface as a monolayer.[20] The saturated adsorption density and the adsorption constant of the five peptides were slightly different. This indicates that several amino acid sequences are effective in the specific adsorption. Because pA and pB have relatively high affinity to the *c* face, the common sequence, ANT, is inferred to be the key factor of the strong interaction.

Fig. 2b shows the adsorption isotherms of the peptides (pAa–pAd and pAr) that were partially or fully modified on the basis of the sequence of pA. The sequence of peptide pAr has the complete opposite of that of pA. The adsorption density of pAr was drastically lowered by comparison with that of pA. A slight decrease in the adsorption density of peptides is found for the partially modified peptides. The sequence of ANT in pA

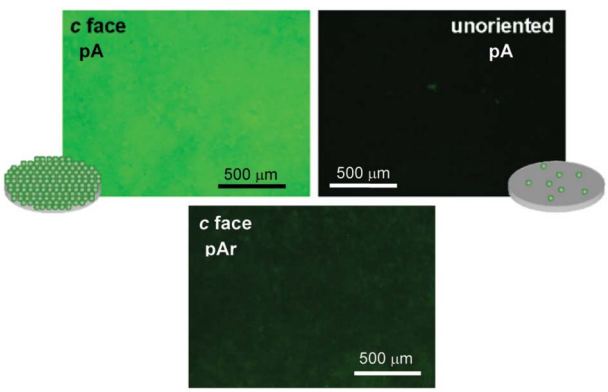

Fig. 1 Fluorescence microscope images and schematic illustrations of the *c* face and unoriented HA pellet with a strongly binding peptide (pA). The image of the *c* face with a weakly binding peptide (pAr) is shown as a reference.

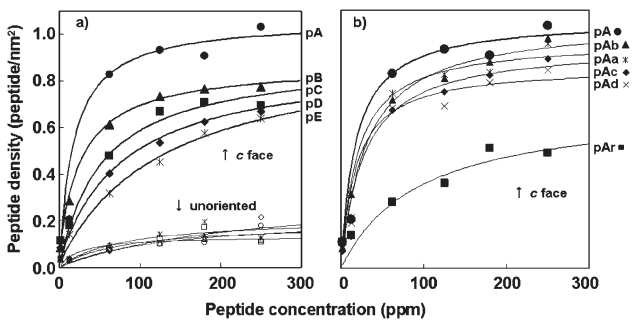

Fig. 2 Adsorption isotherms of peptides on the *c* face and unoriented HA pellets at 25 °C.

was reversed only in pAd, which has the lowest affinity among the partially modified peptides (pAa–pAd). Because all the modified peptides are composed of the same kinds of amino acid, their isoelectric point and the size are the same. The adsorption property of the peptides is thus highly influenced by their local amino acid sequence, ANT. The circular dichroism (CD) band at about 200 nm suggested that only pAr has a different conformation from those of other peptides (Fig. S3, ESI†). The ANT motifs would influence both the functional and conformational properties of the peptides. The amount of pA attaching to the *c* face was decreased by immersion in a saturated NaCl solution. This suggests that the attachment of the aptamer to the specific face is based on the coulombic force.

The epitaxial growth on the *c* face pellets in a supersaturated solution was evaluated to determine the influence of the peptides on the crystal growth. In FESEM and SPM images, a granular structure is observed on the *c* face pellets after growth for 24 h (Fig. 3). The diameters of the grains in the granular structure were 38 and 71 nm in the absence and presence of pA, respectively. The grown surface with pA was relatively flat in comparison to that without the peptide. After washing with purified water, the grown surfaces were immersed in the solution of the *c* face-attaching fluorescent peptide (pA). The fluorescence intensity as shown in Fig. 3c indicates that the surface grown with pA is mainly composed of the *c* face. On the other hand, the surface grown without the peptide is deduced to be covered with (101) because that plane is commonly exposed due to its low surface energy.

All five peptides are concluded to cover the *c* face completely from their saturated amount of adsorption. However, the grain size after epitaxial growth for 24 h depended on the adsorption coefficient as shown in Fig. 4. Interestingly, the crystal growth was not influenced in the presence of pC–pE. This means that the strong binding of the peptides having ANT restricts the crystal growth.

The epitaxial growth of HA is observed on the *c* face-exposing substrate in a supersaturated solution. However, the granular structure or oriented rods are commonly formed on the growing surface. Stable growth of a flat plane would not be achieved due to the relatively low stability of the *c* face. The presence of pA promotes the granular growth with a flat *c* face. According to the isotherm as shown in Fig. 2, *ca.* 90% of the surface is covered with pA at 125 ppm of the peptide concentration. The crystal grows gradually with the ion flux to a small amount of exposed *c* face. Therefore, the Langmuir-type adsorption of the peptide promotes the restricted growth of the *c* face of HA.

Peptides and proteins are known to influence the nucleation of HA in a supersaturated solution through complexation with calcium and phosphate ions. As shown in Fig. S4, ESI†, all the peptides were found to delay the nucleation of HA similarly, although the effects of the peptides on the crystal growth were different. Thus, the restriction of crystal growth with specific peptides cannot be attributed to complexation.

Conclusion

In summary, we have identified artificial peptides that are specific to the *c* face of HA by the m-RNA display method. The adsorption property of the peptides was evaluated from the fluorescence intensity using fluorescein-labeled molecules. The local amino acid sequence, ANT, is assumed to be important for attachment to the *c* face of HA. The presence of the peptides modulates the epitaxial growth in the *c* direction of HA.

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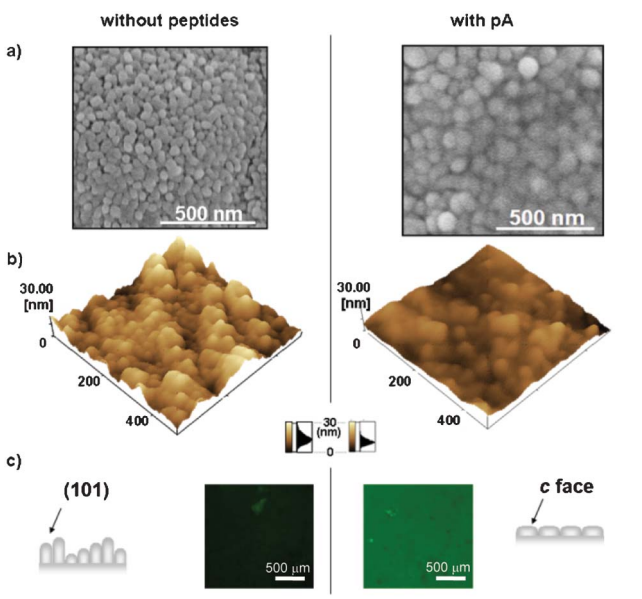

Fig. 3 FESEM (a), SPM (b), and fluorescence microscope (c) images of crystals grown for 24 h in the absence and the presence of pA. Schematic illustrations of the side view of the grown surface.

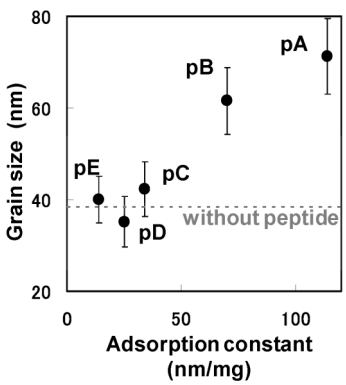

Fig. 4 Grain size as a function of the adsorption constant.

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References

1 A. J. Salgado, O. P. Coutinho and R. L. Reis, *Macromol. Biosci.*, 2004, **4**, 743.
2 C. Tamerler and M. Sarikaya, *MRS Bull.*, 2008, **33**, 504.
3 A. G. Fincham, J. Moradian-Oldak, J. P. Simmer, P. Sarte, E. C. Lau, T. Diekwisch and H. C. Slavkin, *J. Struct. Biol.*, 1994, **112**, 103.
4 J. Kirkham, J. Zhang, S. J. Brookes, R. C. Shore, S. R. Wood, D. A. Smith, M. L. Wallwork, O. H. Ryu and C. Robinson, *J. Dent. Res.*, 2000, **79**, 1943.
5 K. Flade, C. Lau, M. Mertig and W. Pompe, *Chem. Mater.*, 2001, **13**, 3596.
6 P. Ducy, C. Desbois, B. Boyce, G. Pinero, B. Story, C. Dunstan, E. Smith, J. Bonadio, S. Goldstein, C. Gundberg, A. Bradley and G. Karsenty, *Nature*, 1996, **382**, 448.
7 B. Grohe, J. O'Young, D. A. Ionescu, G. Lajoie, K. A. Rogers, M. Karttunen, H. A. Goldberg and G. K. Hunter, *J. Am. Chem. Soc.*, 2007, **129**, 14946.
8 H. Pan, J. Tao, X. Xu and R. Tang, *Langmuir*, 2007, **23**, 8972.
9 T. Matsumoto, M. Okazaki, M. Inoue, Y. Hamada, M. Taira and J. Takahashi, *Biomaterials*, 2006, **27**, 4428.
10 S. Hirakura, T. Kobayashi, S. Ono, Y. Oaki and H. Imai, *Colloids Surf., B*, 2010, **79**, 131.
11 R.W. Roberts, *Curr. Opin. Chem. Biol.*, 1999, **3**, 268.
12 D. Lipovsek and A. Plückthun, *J. Immunol. Methods*, 2004, **290**, 51.
13 K. Shiba, *Curr. Opin. Biotechnol.*, 2010, **21**, 412.
14 M. Sarikaya, C. Tamerler, A. K. Jen, K. Schulten and F. Baneyx, *Nat. Mater.*, 2003, **2**, 577.
15 Y. J. Lee, H. Yi, W. J. Kim, K. Kang, D. S. Yun, M. S. Strano, G. Ceder and A. M. Belcher, *Science*, 2009, **324**, 1051.
16 M. Gungormus, H. Fong, I. W. Kim, J. S. Evans, C. Tamerler and M. Sarikaya, *Biomacromolecules*, 2008, **9**, 966.
17 K. Ohta, M. Kikuchi and J. Tanaka, *Chem. Lett.*, 2003, **32**, 646.
18 T. Kobayashi, S. Ono, S. Hirakura, Y. Oaki and H. Imai, *CrystEngComm*, 2012, **14**, 1143.
19 A. D. Keefe and J. W. Szostak, *Nature*, 2001, **410**, 715.
20 Y. Harpaz, M. Gerstein and C. Chothia, *Structure*, 1994, **2**, 641.