Bioceramics + Soft Material

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Bioceramics x soft material as a simple model to mimic functions in bones

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1. Introduction

Bones are an absolutely essential organ for our body on chemical, biological and mechanical aspects, while the discovering of their hidden functions is challenging because bones, consisting of biominerals and biopolymers, have complicated hierarchical structure. In this review, bioceramics-soft material hybrid materials are introduced as a simplified model material to estimate the functions of bones. The design of polymer is important because it has been considered that such polymer regulates bioceramics formation in the early stage of bone formation. The polypeptide, self-assembling to fine structure, was employed to investigate the effect of functional group pattern in angstrom scale on hydroxyapatite (HAp) mineralization. Also, polymeric gels were applied to investigate the energy dissipation coming from mineral deformation and the effect of anisotropic space of polymer on HAp mineralization because the polymer network in gel can be deformed by macroscopic deformation. Furthermore, the bioceramics-tough gel system itself can be used as biomaterials bondable to bones. The hybridization of bioceramics and soft material has great potential to estimate various functions and mechanisms of bones up to material design.

Key-words : Mineralization, Hydroxyapatite, Hydrogel, Sacrificial bond

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For an application of HAp mineralized tough gel, in vivo robust fixation method was introduced. The tough gel materials, expected as an artificial cartilage, were difficult to be fixed in vivo because the watery surface did not accept any medical glues. The HAp hybridization on the surface of gel gifts the gel matrix osteoconductive property. Therefore, the HAp mineralized gel induced bone formation into gel matrix, resulting unified structure between bone and gel.

The last section is future prospect of the HAp-polymer materials. As mentioned above, the bones must have many uncovered functions yet. Up to material design, the novel functions will contribute to develop functional materials.

2. CaP mineralization regulated by positional pattern of nucleation site

In the earliest stage of HAp formation during biomineralization process, a biomacromolecule such as collagen works as nucleation site and regulates subsequent mineral growth. The nucleation is attracted by the functional group of protein and polysaccharide because the mineral source ions are attractively captured by electrostatic interaction. Tanahashi et al. well-revealed effective functional groups upon HAp mineralization by using the terminal-functionalized self-assembled monomer (SAM). Among various functional groups, the negative charged groups like carboxyl or phosphate groups showed the most efficient mineralization of HAp from simulated body fluid, resulting that the capture of calcium ion is an important trigger to start the HAp mineralization event. Sato also mentioned that the carboxyl groups capture the calcium ion in advance of HAp nucleation. These facts convince why the collagen and osteopontin presenting carboxyl groups act as a biomineralization template. Accompanying with these mineralization experiments, the effect of spatial position of functional group on biomineralization must be further curiosity. The collagen triple helix and its higher-order architecture show regulated pattern of functional groups in angstrom scale due to secondary and tertiary structure formation as an intrinsic property of proteins. Even if the SAM of surfactant molecules ideally form the hexagonal packing structure, the SAMs usually possess defect structure. To investigate the effect of functional group pattern, a polypeptide was employed as the HAp mineralization substrate. Polypeptide is compositionally same with protein while usually consist of shorter amino acid sequence than that of protein. In the present, the amino acid sequence can be almost completely controlled by chemically or biologically synthetic methods. Therefore, the self-assemble structure of polypeptide can be designed, namely, the spatial position of functional groups can be regulated in angstrom scale. To consider the effect of spatial position simply, β-sheet polypeptide, self-assembling to two-dimensional monolayer, was designed. The Fig. 2(a) shows the common architecture of polypeptide in this study. The alternative sequence of hydrophobic (green) and hydrophilic (red) amino acids enables to fold β-sheet structure. The red part is freely chosen from glutamic acid (E) with carboxyl group, lysine (K) with amino group or valine (V) with electrostatically inert group. The polyethylene glycol (PEG), introduced to the polypeptide terminal, works as a spacer between peptide self-assembled nanofibers. The polypeptides with PEG unit were chemically synthesized by solid phase peptide synthesis. When the hard substrate (mica) for scanning electron microscopy (SEM) and carbon grid for electron diffraction (ED) were soaked in the polypeptide solution overnight with proper concentration and pH, the polypeptides spontaneously formed anti-parallel β-sheet structure and subsequent large monolayer on the substrate [Fig. 2(b)]. The displayed patterns of functional groups in various polypeptides were shown in Fig. 2(c). Then, the calcium phosphate (CaP) mineralization was performed upon these substrates by an alternating soaking method. A droplet of 50 mM calcium acetate solution was placed on the substrate for 1 min. Then the substrate was rinsed by pure water. In the same way, the droplet of 30 mM ammonium phosphate dibasic solution...
was placed for 10 min, and the substrate was washed and dried. First, comparing the bare mica with the (LE)₈, the CaP mineralization with random form was observed on the bare mica, while the unidirectionally orientated CaP was realized on (LE)₈. Interestingly, even though the CaP on (LE)₈ was amorphous phase showing halo in ED image, the anisotropic growing was achieved. It indicated that the CaP mineralization is strongly controlled by the orientation of functional groups of template polymer. When the pattern was changed, the (LELK)₄ and (VEVV)₄ showed unidirectionally oriented HAp; (VEV₆)₄ showed amorphous dots; and (LK)₈ showed no precipitation. In comparison of (LE)₈, (VEVV)₄ and (VEV₆)₄, the density of carboxyl groups affected to morphology and crystal phase of mineral. Especially, there may be proper position of carboxyl group like (VEVV)₄ and (LELK)₄ to form HAp crystal. In comparison of (LE)₈, (LELK)₄ and (LK)₈, the amino group of lysine decreased efficiency of CaP mineralization, agreed with the past SAM report. In summary of the polypeptide system, the spatial position of functional groups is one of important factors to determine the mineral morphology and crystal phase in the early stage of mineralization. Not only the CaP mineral but also such controlled mineralization of other inorganic matters like titanium dioxide, silicon dioxide and calcium carbonate were realized.

Fig. 2. (a) Common structure of polypeptide and chemical structures of amino acids and PEG. The displayed structure is (LE)₈. (b) Self-assembly of β-strand polypeptide to anti-parallel β-sheet monolayer. (c) Functional group patterns of various polypeptides.

Fig. 3. SEM and ED images of CaP on (a) bare mica, (b) (LE)₈, (c) (LELK)₄, (d) (LK)₈, (e) (VEVV)₄, (f) (VEV₆). ED images were obtained from the samples prepared on carbon grid. Inset numbers were Miller index of HAp. Figure adapted with permission from Refs. 4) and 5).
3. Hydrogel toughened by bony sacrificial bond

In molecular scale of bone structure, the HAp crystals are aligned between the collagen bundles. Imaging the crack propagation to such hybrid structure, energy dissipation systems should be simply classified to three terms: HAp crystal fracture, collagen helix uncoiling and delamination between HAp and collagen. Among them, the HAp crystal fracture working as a sacrificial bond during fracture event was introduced in this section. To simplify the bone structure, the HAp-polymeric hydrogel composite as a model was designed [Fig. 4(a)].

To avoid the electrostatic interaction between polymer and HAp, to avoid contribution of delamination between HAp and polymer in other words, non-charged and neutral polymer, poly(dimethylacrylamide) (PDMAAm), was employed. First, the PDMAAm gel was synthesized as a reaction field of HAp mineralization. The HAp mineralization was performed in the PDMAAm gel. Usually, the HAp polycrystals were mostly formed in the gel matrix (not gel surface) and were much larger than the mesh size of PDMAAm network. In the result, the HAp was physically entangled with polymer network, meaning that this PDMAAm network can transfer force to HAp polycrystals. This state is called as HAp hybridization in gel matrix. Finally, the other PDMAAm network as the second network was introduced in the HAp/PDMAAm gel to obtain HAp/PDMAAm gel. The second PDMAAm network was interpenetrated into HAp hybridized PDMAAm network but was independent from HAp polycrystals. Specifically, the first PDMAAm gel was synthesized by the redox polymerization of the aqueous solution containing 1 M DMAAm monomer, 2 mol% (referred to monomer) crosslinker, 0.1 mol% (referred to monomer) thermal initiator, and 1.0 x 10^{-4} vol% accelerator. The HAp mineralization was done by the alternative soaking of the PDMAAm gel in 300 mM dipotassium phosphate and 500 mM calcium chloride solution up to 7 cycles (n: cycle number). The HAp hybridized PDMAAm gel was soaked in the monomer solution containing 2 M DMAAm, 0.2 mol% crosslinker, and 0.1 mol% initiator overnight, and 2nd PDMAAm network was thermally polymerized. Figure 4(b) showed the photographs of pristine DN gel without HAp mineralization (n = 0) and HAp/PDN gel (n = 7). The HAp was homogeneously distributed in gel matrix. When the gel was stretched in tensile test [A: stretch ratio, (initial length + displacement)/initial length], the modulus, strength and maximum strain were obviously enhanced with increasing in HAp fraction [Fig. 4(c)]. Additionally, the hysteresis loss, comparable to dissipated energy, was measured from load-unloading test, and the dissipated energy was divided into polymer and HAp contributions [Fig. 4(d)]. In the result, the HAp was major contribution in the total dissipated energy and was 30-fold higher than polymer in maximum. To reveal the origin of the energy dissipation of HAp, the HAp polycrystal morphology was observed by transmittance electron microscope (TEM) [Fig. 4(e)]. The HAp had the spherical and polycrystalline form before stretching, while it was deformed to the oval sphere at the stretched state. In higher magnification, the connecting point between monocystaline HAp was amorphous phase, and this point was bent instead of HAp crystal region because the amorphous region will be softer than crystal phase. Therefore, the origin of energy dissipation of HAp was deformation of the amorphous region. Actually, the dissipated energy was inverse-proportional to crystallinity in the crystallinity-modulated samples. These results suggest that the low-crystalline HAp crystals are distributed in the gel matrix. The first PDMAAm gel was synthesized by the redox polymerization of the aqueous solution containing 1 M DMAAm monomer, 2 mol% (referred to monomer) crosslinker, 0.1 mol% (referred to monomer) thermal initiator, and 1.0 x 10^{-4} vol% accelerator. The HAp mineralization was done by the alternative soaking of the PDMAAm gel in 300 mM dipotassium phosphate and 500 mM calcium chloride solution up to 7 cycles (n: cycle number). 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Fig. 4. (a) Schematic image of HAp/PDN gel. The first PDMAAm network is reaction field of HAp mineralization. The HAp polycrystals are larger than the mesh size of the PDMAAm network, resulting that the PDMAAm network can transfer force to HAp. The other PDMAAm network is interpenetrated into the HAp/PDMAAm gel. (b) Photographs of Pristine DN gel (n = 0) and HAp/PDN gel (n = 7). (c) Stress strain curves of HAp/PDN gels with different HAp fractions. (d) Dissipation energies of HAp and polymer. (e) TEM images of HAp polycrystals at stretch ratio λ = 1 and 2. Figure adapted with permission from Ref. 6).
crystallinity of HAp in bone might be rational design for energy dissipation system in molecular scale. Additionally, there is a critical range of modulus contrast between polymer and mineral to work as sacrificial bond. If stiffer polymer is hybridized with HAp, the HAp crystal might work as sacrificial bond.

4. HAp mineralization in anisotropic reaction field

HAp in bones is well-oriented along the collagen bundles. In the earliest stage of biomineralization, it has been suggested that template polymer is self-assembled at first, and then mineralization occurs from solute ions on the polymer. Therefore, the geometry of polymer in the initial state will determine the mineralized HAp. To investigate the shape regulation by polymer geometry, HAp mineralization was carried out in the hydrogel under stretched state (Fig. 5).11) This experiment assumed the affine deformation of polymer network, meaning that the deformation ratio in macroscopic gel is equal to microscale deformation of polymer network. The high-stretchable DN gel was employed as the mineralization field. The DN gels consist of interpenetrating structure of polyelectrolyte network and neutral polymer network. The former network works as mineralization site, while such polyelectrolyte gets shrink in the mineralization solution due to ionic osmotic pressure. To prevent volume change, the neutral polymer network with ionic-inert feature is combined as a support network. The neutral polymer network is expected to maintain original volume during mineralization. The poly(2-acrylamido-2-methylpropane-sulfonic acid) (PAMPS) and Poly(acrylamide) (PAAm) were selected as polyelectrolyte and neutral polymer, respectively. The PAMPS/PAAm DN gel was synthesized from two-step radical polymerization. In the general protocol, the solution containing 1 M AMPS, 2 mol% cross-linker, and 0.1 mol% UV initiator was polymerized by UV irradiation. The PAMPS gel was soaked in the second solution of 2 M AAm, 0.02 mol% cross-linker, and 0.01 mol% UV initiator overnight. Then the second PAAm network was polymerized in the presence of first PAMPS network. The HAp mineralization in the stretched DN gel was performed by the alternative soaking method that is the same way to section 3. The orientation of mineralized HAp was measured by wide angle synchrotron X-ray diffraction [Fig. 5(a)]. At unstretched state (\( \lambda = 1 \)), Debye–Scherrer ring was observed indicating that HAp was isotropic polycrystals. At the stretched state (\( \lambda = 4 \)), the crescent arcs assigned to 002 and 004 of HAp were shown, meaning that the c-axis of HAp was aligned to stretched direction. The orientation degree calculated from the diffraction profiles was increased with increasing the stretch ratio and got close to that of natural bone, rabbit tibia [Fig. 5(b)]. To identify the morphology of HAp, the TEM observation was performed. The HAp polycrystal showed the isotropic sphere at unstretched state (\( \lambda = 1 \)) while formed the oriented morphology along the stretched direction at \( \lambda = 4 \) [Fig. 5(c)]. From these results, the initial space made from polymer network determines the morphology of mineral. It implies that the collagens will self-assemble to the bundle structure before HAp biomineralization, resulting that the oriented HAp is mineralized in bone.

5. Fixation of gel materials to bone via osteogenesis penetration

Beyond conventionally mechanical weak gels, various tough hydrogels have been developed past 20 years.21)-23) Among them, the mechanical properties get close to the same level of industrial rubbers. Such tough gel has been a promising material to be applied to load-bearing biomat-eriaIs. The cartilage replacement is the most expected application because the cartilage regeneration is still challenging remedy. The cartilages contain few blood vessels and cells so that they are “eraser”-like tissue, meaning non-regenerable in general. The critical issue of the gel application to artificial cartilage is the robust fixation in vivo. The major composition of gel materials is water, not accepting any medical glues. Also, suturing is not suitable for gel material because the suture thread easily tears even tough gel matrix because of large stress concentration of

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**Fig. 5.** (a) Wide angle X-ray diffraction images and 1D profiles of HAp/DN gel at different stretched states (\( \lambda = 1, 4 \)). (b) Orientation degree of 002 plane of HAp as function of elongation ratio. (c) TEM images of HAp nanocrystals in different stretched states. Figure adapted with permission from Ref. 11).
the thread. In this section, a robust fixation of tough DN gel to bone in vivo was introduced utilizing HAp mineralization. The gel has both liquid and solid features. Even though the matrix is non-fluidic solid, the substance diffusion like liquid is acceptable. Therefore, the osteo-inert DN gel surface can be easily modified to the osteo-conductive by HAp mineralization [Fig. 6(a)]. To evaluate the bonding capability between the DN gel and bone, the HAp/DN gel and pristine DN gel were implanted into a bone defect of rabbit femur. The cylindrical DN gel plug was made, and the side wall of cylinder was hybridized with HAp by the alternative soaking. This plug with a protector gel was implanted in the defect created at the rabbit femur [Fig. 6(b)]. After the implantation periods, the gel with surround bone was harvested, and the sponge bone opposite to the operated site was removed up to protector gel to make a pass for biomechanical test. The bonding strength was measured by a push-out test [Fig. 6(b)]. The pristine gel showed the ~4 N in all implantation periods corresponding to the friction force [Fig. 6(c)]. On the other hand, the HAp/DN gel showed effective bonding at 2 weeks, and the bond strength finally exceeded the strength of DN gel matrix at 4 weeks. From the TEM observation of the boundary region between gel and bone, the osteogenesis penetration into gel matrix region was confirmed [Fig. 6(d)]. The synthetic HAp polycrystals were fused with the immature bone. Therefore, the formation of unified boundary structure contributed the robust fixation of gel to bone. This method was also effective to a non chemically synthesized hydrogel consisting of biomacromolecules like collagen, meaning that it is universal method for solid and permeable hydrogel materials and does not affect to gel bulk properties.

Associating with the implantation of HAp/DN gel in vivo, two advanced experiments were designed. One is the more rapid fixation of DN gel than HAp hybridized DN gel. HAp associating fixation utilizes osteogenesis penetration into gel matrix. The biological event takes at least 2 weeks in the case of rabbit model. In the clinical application for human, it will take more. For the better QOL, the effective fixation in shorter period is expected. Compared with the biological reaction, a chemical reaction must be much faster. In physiological condition, the HAp is the most stable crystal phase in calcium phosphate, and metastable phases gradually transform to HAp in body. Utilizing the chemical equilibrium, the metastable calcium phosphate, monetite, was hybridized with the outermost layer of DN gel for initial pre-fixation in advance of osteogenesis penetration [Fig. 7(a)]. The same PAMPS/PDMAAm DN gel was used. In the method of monetite hybridization to the outermost layer of the gel surface, the DN gel was soaked in 500 mM dipotassium phosphate solution for 1 day. Then the gel surface was washed by pure water. Next the gel was soaked in 500 mM calcium acetate solution for 1 day and was rinsed again. Finally, the gel was thermally annealed at 121 °C for 20 min to obtain monetite. When the monetite/DN gel was implanted in the same way to HAp/DN gel research, the effective bonding strength (4N<) was detected one week earlier than HAp/DN gel. For this one week, the monetite was gradually dissolved to ions, and then HAp was recrystallized at the vacant space between gel and bone. This accumulation increased physical interlock of gel in bone defect, resulting that the monetite DN gel showed increment of fixation.

Fig. 6. (a) Fixation of DN gel to bone utilizing HAp hybridization. (b) Geometry of push-out test to evaluate bonding strength. (c) Bonding strength of DN and HAp/DN gels at 1, 2, 4 and 12 weeks after implantation. (d) TEM images of surface of HAp/DN gel and boundary between HAp/DN gel and bone at 4 weeks with schematic image of osteogenesis penetration. Figure adapted with permission from Refs. 12) and 13).
Fig. 7. (a) Bonding strength of monetite/DN gel and HAp/DN gel at 3, 7 and 14 days after implantation. (b) ${^{44}\text{Ca}}/{^{40}\text{Ca}}$ ratio image of boundary of bone and $^{44}\text{HAp}$/DN gel at 2 and 4 weeks. Figure adapted with permission from Refs. 25) and 26).

The second experiment is the direct observation of reutilization of HAp implant to osteogenesis phenomenon. In clinical application, calcium phosphate materials like HAp and $\beta$-TCP have been used for long time. Mainly, their implantations are expected to help bone regeneration because of same inorganic component with bones. However, reutilization of these material to bone formation was difficult to prove because both the implant and bone have same inorganic component. To resolve this problem, the stable isotope doping was employed.$^{26}$ When the HAp was mineralized by the alternative soaking method, the stable calcium isotope ($^{44}\text{Ca}$) was added to regular calcium chemical to modulate isotope ratio. The isotope doped HAp ($^{44}\text{HAp}$)/DN gel was implanted at the bone defect of rabbit femur, and the isotope imaging was carried out by using the isotope microscopy developed by Yurimoto et al.$^{27}$ [Fig. 7(b)]. At the boundary between bone and $^{44}\text{HAp}$/DN gel, the isotope ratio of ${^{44}\text{Ca}}/{^{40}\text{Ca}}$ was 0.020 at the bone region that is natural isotope ratio, while the gel region showed much higher value of 0.09 for two weeks after implantation. At 6 weeks, the region of newly formed bone showed above 0.02 ratio, meaning that this new bone was formed using the implanted $^{44}\text{HAp}$. This is the strong evidence to directly identify the re-utilization of HAp in osteogenesis.

6. Future prospects

The fusion of HAp and polymer is one of ideally simplified models to investigate functions and forming mechanism of complicated bones. In the earliest stage of bone formation, self-assembled polymer substrates exist at first, and then CaP mineralization occurs associated with the substrates. Considering this process, the polymers are an important role to determine fate of CaP like crystal phase and morphology. The polypeptide can self-assemble to monolayer displaying regulated pattern of functional groups in angstrom scale and evaluate the effect of spatial position of functional groups on mineralization. Polymeric hydrogels can be deformed in macroscale. Simultaneously, the polymer network is stretched in microscale, too. Therefore, the initial polymer geometry can be regulated to evaluate the effect on mineralization. Also, the CaP nanocrystals hybridized in gel can be deformed via network deformation. Not only for the investigation of bones, but the hybrid material itself has a potential for clinical application. For the robust fixation of gel materials in vivo, the CaP hybridized to the outermost surface of hydrogel can induce ingrowth of bone into gel matrix, resulting the strong and non-toxic fixation beyond the strength of gel matrix.

Probably, the bones still have many secrets in functions based on the complicated structure. The combination of calcium phosphate and soft material with a design extracting important essence has potential to uncover novel functions of bones. For example, when an acidic polymer is combined with HAp, the contribution of the delamination between HAp and polymer will be evaluated. Also, comparing collagen and gelatin matrix, the contribution of uncoiling of collagen triple helix will be identified. Utilizing stretchability of macroscopic gel, the entropy of polymer network can be tuned. The effect of entropy on bio-mineralization must be evaluated. The combination of bioceramics and soft material will be powerful model to clarify bone mechanisms up to your material design.

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