Bone engineering by phosphorylated-pullulan and β-TCP composite

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Abstract

A multifunctional biomaterial with the capacity bond to hard tissues, such as bones and teeth, is a real need for medical and dental applications in tissue engineering and regenerative medicine. Recently, we created phosphorylated-pullulan (PPL), capable of binding to hydroxyapatite in bones and teeth. In the present study, we employed PPL as a novel biocompatible material for bone engineering. First, an *in vitro* evaluation of the mechanical properties of PPL demonstrated both PPL and PPL/β-TCP composites have higher shear bond strength than materials in current clinical use, including polymethylmethacrylate (PMMA) cement and α-tricalcium phosphate (TCP) cement, Biopex-R. Further, the compressive strength of PPL/β-TCP composite was significantly higher than Biopex-R. Next, *in vivo* osteoconductivity of PPL/β-TCP composite was investigated in a murine intramedular injection model. Bone formation was observed 5 weeks after injection of PPL/β-TCP composite, which was even more evident at 8 weeks; whereas, no bone formation was detected after injection of PPL alone. We then applied PPL/β-TCP composite to a rabbit ulnar bone defect model and observed bone formation comparable to that induced by Biopex-R. Implantation of PPL/β-TCP composite induced new bone formation at 4 weeks, which was remarkably evident at 8 weeks. In contrast, Biopex-R remained isolated from the surrounding bone at 8 weeks. In a pig vertebral bone defect model, defects treated with PPL/β-TCP composite were almost completely replaced by new bone; whereas, PPL alone failed to induce bone formation. Collectively, our results suggest PPL/β-TCP composite may be useful for bone engineering.

1. Introduction

Bone cements are used to anchor artificial joints, with polymethylmethacrylate (PMMA) cement being widely utilized for its strong mechanical properties [1, 2]. Fixation of components with PMMA cement during total joint replacement represents the most common clinical application, followed by use in osteosynthesis, tumor surgery, percutaneous vertebroplasty and treatment of musculoskeletal infections with combined use of antibiotics [3, 4]. However, PMMA cement is not bioactive or absorbed, meaning PMMA can potentially cause adverse effects as it remains in the body for an extended duration [5].

Ideally, bone cement needs to be biocompatible, biodegradable and capable of being formed into desired shapes. Some of the most promising biocompatible substitutes are calcium phosphate (CP)-based materials, including hydroxyapatite, tricalcium phosphate (TCP) and their combination. CP cements are non-toxic, biodegradable and, most importantly, integrated into the tissue [6]. However, CP cements are fragile with weak mechanical properties [7]; although, many researchers have modified CP to improve mechanical properties and *in vivo* absorption velocity for clinical applications [8-10].

Phosphorylated functional monomers have successfully been used as dental adhesives to bond composites to tooth structures [11-14]. Recently, we developed phosphorylated-pullulan (PPL) capable of chemically bonding to bones and teeth [15, 16]. Pullulan (PL) is a non-ionic, non-toxic, non-immunogenic, non-carcinogenic, non-mutagenic polysaccharide produced by the polymorphic fungus *Aureobasidium pullulans* [17]. PL exhibits low viscosity, which is stably maintained after exposure to heat and/or pH changes. The unique linkage of α (1→4) and α (1→6) in PL underlie its distinctive adhesive properties [17]. In the present study, we evaluated *in vitro* mechanical properties of PPL and PPL/β-TCP composites before administration into mice femurs to assess *in vivo* biocompatibility and osteogenic ability. Next, the composite was applied to a bone defect model; with bone formation being compared to clinically used α-TCP cement, Biopex-R. Finally, PPL/β-TCP composite was applied to a load-bearing vertebral body defect model. Our results suggest PPL/β-TCP composite may be useful for bone engineering.

2. Experimental procedures

2.1. Phosphorylated-pullulan (PPL)

PPL was developed, as described previously [15]. In brief, a 1% aqueous phosphate solution (pH 5.5) was added to pharmacopeial PL (Hayashibara Shoji, Okayama, Japan), dissolved into distilled water and stirred for 1 h at room temperature (RT). This solution was allowed to evaporate at 100°C under reduced pressure, kept at 170°C for 5 h and then cooled to RT. The resultant product was dissolved in distilled water, ultrafiltrated and freeze-dried. Degree of phosphorylation was analyzed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES; VISTA-PRO, Seiko Instruments, Chiba, Japan). Molar quantity of the phosphate group in PPL content was calculated from phosphorus data of ICP. Molar quantity of the maltotriose unit was calculated by subtracting phosphate group mass and sodium mass from total PPL mass. One maltotriose unit includes nine hydroxy groups. Degree of substitution was calculated by the formula indicated below.

$$\text{degree of substitution} = \frac{\frac{P}{31}}{9 \times \frac{M - Na\frac{22}{23} - P\frac{80}{31}}{486}}$$

M: sample mass, P: phosphorus mass from ICP data, Na: sodium mass from ICP data, 31: atomic weight of phosphorus, 486: molar mass of maltotriose unit, 80: molar mass increased by substituting hydroxy group to phosphate group, 22: molar mass increased by substituting hydroxy group of phosphate group to sodium salts, 23: atomic weight of phosphorus, 9: hydroxy group number in the maltotriose unit.

2.2. Nuclear magnetic resonance (NMR) spectrometer

^{1}H NMR spectra were obtained using a Varian 400MR (Varian, CA). PL and PPL were dissolved in deuterium oxide (D_2O) at a concentration of 1%. Trimethylsilyl propanoic acid (TMSP, Sigma-Aldrich, MO) was used as an internal reference for aqueous solvents, D_2O.

Samples were measured 16 times in the frequency band of 400 MHz. VNMRJ 3.0 (Varian) was used for data acquisition and Mnova software (Mestrelab Research, Santiago de Compostela, Spain) was used for data processing.

2.3. Shear bond strength

Pure titanium plates (Grade II, 10×10 mm, 3-mm-thick, Kobe Steel, Kobe, Japan) and hydroxyapatite (APP-601, Pentax, Tokyo, Japan; 13 mm in diameter, 1-mm-thick) were used to measure shear bond strength. Each plate was embedded in a slow-setting epoxy resin (Epofix Resin, Struers, Denmark) and plate surfaces were ground using 1000-grit silicon carbide paper under water-cooling. Plates were ultrasonically cleaned in ethanol for 10 min and dried at RT. Subsequently, titanium and hydroxyapatite rods (5 mm in diameter) were bonded onto the prepared titanium and hydroxyapatite plates using α-TCP cement Biopex-R (Taisho Pharmaceutical Co. Ltd., Tokyo, Japan), β-TCP (Taihei Chemical Industrial Co., Ltd., Osaka, Japan), PMMA cement (Depuy Synthes, West Chester, PA), PPL or PPL/β-TCP composite, respectively. PMMA cement was prepared by mixing PMMA powder with liquid methylmethacrylate (MMA). β-TCP (1 g), PPL (1 g) or PPL/β-TCP (1g each) was kneaded with 1 ml of 1M $CaCl_2$. Ratio of PPL and β-TCP (1:1) was determined by adhesive force of the composite, which increased with %β-TCP content to reach a maximum rate of 50% (figure S1). A composite of >50% β-TCP had a coarse texture and was not suitable for use in the current study. Shear bond strength was determined using a universal testing machine (AGS-1000A, Shimadzu Co., Japan) with a crosshead speed of 0.5 mm/min. Five specimens were tested for each group. Data between two groups were analyzed using a Student's t-test. *P*-values of <0.05 were considered statistically significant.

2.4. Compressive strength

Biopex-R, β-TCP, PPL and PPL/β-TCP composite were used. β-TCP (1 g), PPL (1 g) or PPL/β-TCP (1g each) was kneaded with 1 ml of 1M $CaCl_2$. Samples were set into a mold (diameter of 5 mm and thickness of 10 mm) and consolidated. The compressive strength of

each sample was determined using a universal testing machine (AGS-1000A, Shimadzu Co., Kyoto, Japan) with a crosshead speed of 0.5 mm/min. Four specimens were tested for each group. Data between two groups were analyzed using a Student's t-test. *P*-values of <0.05 were considered statistically significant.

2.5. Animals

C57BL/6J mice (female, 6–8 weeks) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Female New Zealand white rabbits weighing 2.0–2.5 kg were obtained from Shimizu Laboratory Supplies). Pigs (female, 30 kg) were from Okayama JA Chikusan Co., Ltd. (Okayama, Japan). Animals were housed in a temperature-controlled environment with a 12 h light/12 h dark cycle and allowed free access to water and food. The Animal Care and Use Committee at Okayama University approved all animal experiments conducted in this study.

2.6. Intramedular injection model

Mice were anesthetized with ketamine (1 mg/kg) and sodium pentobarbital (40 mg/kg). The distal femur was exposed and the bone-marrow space was drilled through the intercondylar notch with a 24-gauge needle. PPL alone (1 g) or PPL/β-TCP (1 g each) were kneaded with 1 ml of 1M $CaCl_2$ and injected into the medulla of the femur. Skin was closed using 5-0 nylon sutures and mice were allowed full weight bearing without any joint immobilization. Mice were sacrificed at 2, 5 and 8 weeks after injection (three mice at each time point). Femurs were resected, fixed in 10% phosphate-buffered formalin, decalcified in 10% formic acid, embedded in paraffin and cut in transverse sections at the diaphysis. Tissue sections were stained with hematoxylin and eosin, as well as Periodic Acid-Schiff (PAS) stain. In some mice, femurs were scanned by micro-CT (Latheta LCT-200, Hitachi Aloka Medical, Tokyo, Japan) using 48 μm slices (0.3 mm interval), with bone mineral density (BMD) of individual trabecular bone area calculated by accompanying image-analyzing software, LaTheta v1.20.

2.7. Ulnar defect model

Rabbits were anesthetized with ketamine (1 mg/kg) and isoflurane (0.25–5 L/min). Forearms were shaved and draped in a sterile fashion, and an incision was made above the ulna. A 10 mm bone defect was made in the middle of the ulna using a bone saw, and this defect was randomly filled with Biopex-R or PPL/β-TCP (1 g each kneaded with 1 ml of 1M $CaCl_2$). Untreated defects were used as controls. Rabbits were then housed in separate cages without any immobilization and sacrificed at 4 and 8 weeks after surgery (three rabbits each). Forearms were removed and scanned by micro-CT using 48 μm slices (0.3 mm interval). Subsequently, forearms were fixed in 4% paraformaldehyde, decalcified in 10% EDTA, embedded in paraffin and sections were stained with hematoxylin and eosin, as well as Safranin O.

2.8. Vertebral body defect model

Pigs were anesthetized with intramuscular atropine (0.05 mg/kg), ketamine (5 mg/kg), medetomidine (40 μg/kg) and midazolam (0.2 mg/kg). Pigs were endotracheally intubated and maintained on isoflurane (2%) with mechanical ventilation (15 breaths/min; tidal volume, 10 ml/kg). Antibiotics were administered before surgery. Hydration was maintained with lactated Ringer solution. Pigs were laid on an operating table in a right lateral decubitus position. A left retroperitoneal approach was performed through a transverse incision in the left lower quadrant of the abdomen. After retracting the iliopsoas muscle, a partial corpectomy was performed in a 1 cm square at L2, L3 and L4 levels using a bone saw. Either Biopex-R or PPL/β-TCP (1 g each kneaded with 1 ml of 1M $CaCl_2$) was applied to bone defects. Untreated defects were used as controls. Pigs were sacrificed at 4 and 8 weeks after surgery and the spines were resected (two pigs each). Vertebras were scanned by μ-CT using 48 μm slices (0.3 mm interval). Regions of interest were set on femurs and CT number (HU: Hounsfield Unit) was analyzed by AZE VirtualPlace software (AZE, Ltd, Tokyo, Japan). Femurs were then fixed in 10% formalin, decalcified in 10% EDTA and embedded in paraffin. Sections were stained with hematoxylin and eosin.

3. Results

3.1. NMR spectra

Figure 1(a) shows the chemical structure of PPL; phosphorylation degree of PLL was 6.9%. ^{1}H NMR spectrum for PL and PPL is shown in figure 1(b). Signal present at 4.7 ppm was caused by residual HDO in the D_2O solvent. Signals around 4.9 and 5.4 ppm were anomeric protons internal to the polymer chain. PL was characterized by three envelope peaks (figure 1(b), brackets) resonating between 3.3 and 4.0 ppm [18]. In the PPL chain, methylene groups bound to phosphate groups were randomly distributed, therefore, different phosphorylation positions and degrees of substitution occurred in PPL. This resulted in the shoulder peak from 3.9 to 4.1 ppm (figure 1(b), arrowheads). Glucose and glucose-6-phosphate spectra are shown for reference.

3.2. *In vitro* properties of PPL

The shear bond strength of PPL to titanium and hydroxyapatite was significantly higher than PMMA (figure 2(a)). Surprisingly, some of the hydroxyapatite plates were wrecked without coming unglued at the bonding plane to the hydroxyapatite rod (figure S2). Shear bond strength of PPL to titanium and hydroxyapatite was maintained in the presence of β-TCP. No strength was observed with either Biopex-R or β-TCP alone (figure 2(a)). Compressive strength of consolidated PPL did not significantly differ from Biopex-R; however, PPL/β-TCP composite demonstrated a significant 1.7-fold increase relative to Biopex-R (figure 2(b)). No compressive strength was observed for β-TCP alone. TPPL/β-TCP composite exhibits excellent mechanical properties including shear bond strength of 13-16 MPa and compressive strength of 56-76 MPa; thus, PPL/β-TCP composite appears suitable for use in bone engineering applications.

3.3. Bone formation by PPL/β-TCP composite

To assess whether PPL/β-TCP composite could be applied to bone engineering *in vivo*,

PPL/β-TCP composite was injected into mice femurs (PPL alone was employed as a control). Two weeks after injection, bone marrow cells surrounded the defect in a circular pattern (figure 3(a)-(i)(ii), arrows). A PAS-positive layer was observed along the periphery of bone defects in both groups (figure S3, arrowheads), suggesting PPL was still present in defects at 2 weeks. However, PPL was gradually replaced by bone marrow at 5 weeks (figure 3(a)-(iii)), indicating PPL was absorbed by this time. Notably, bone formation was observed 5 weeks after injection of PPL/β-TCP composite (close-up image of figure 3(a)-(iv), arrowheads), which was even more evident at 8 weeks (close-up image of figure 3(a)-(vi), arrowheads). No bone formation was observed in mice treated with PPL alone, even at 8 weeks (figure 3(a)-(v)). We then assessed BMD at 4 weeks after the injection by measuring micro-CT images (figure 3(b)). BMD in PPL/β-TCP composite groups was higher than was seen in defect and PPL groups. There was no difference between defect and PPL groups (figure 3(c)).

These results prompted comparison of bone formation capability between Biopex-R and PPL/β-TCP composite. Our assessment employed a rabbit bone defect model (figure 4(a)) with radiographic techniques to evaluate the postoperative course. Biopex-R completely filled bone defects by 4 weeks post-operation (figure 4(b) left). However, increased translucency was observed in between Biopex-R and host bone at 8 weeks (figure 4(b) left, closed arrowheads), indicating an apparent gap between cement and bone. Macroscopically, Biopex-R remained isolated from existing bone (figure 4(c) left, arrows). Conversely, implantation of PPL/β-TCP composite induced new bone formation at 4 weeks, a feature that was remarkably evident at 8 weeks (figure 4(b) right, open arrowheads). Further, a cut section revealed the defect was completely recovered by bone healing (figure 4(c) right, circle). Histologically, fibrous tissue circled Biopex-R at 4 weeks post-implantation (figure 5(a), arrowheads) with fragmented bone ingrowth observed in the intervening space (figure 5(b), asterisk). Interestingly, PPL/β-TCP composite induced hyaline cartilage-like cells at 4 weeks (figure 5(c)) and evidence of mature bone tissue at 8 weeks (figure 5(d)), indicating new bone was formed by endochondral ossification.

To further examine osteogenic capability, we implanted PPL/β-TCP composite in a pig

vertebral body defect model. Neither untreated defects nor Biopex-R treated defects demonstrated any bone formation as assessed by μ-CT; whereas, defects implanted with PPL/β-TCP composite were replaced by new bone (figure 6(a), arrowheads). CT number was significantly higher in PPL/β-TCP composite groups than that in defect controls (figure 6(b)). Histologically, defects treated with PPL/β-TCP composite indicated new bone formation (figure 7(c), arrowheads) around existing bone (figure 7(c), asterisks) at 4 weeks. Trabecular bone was formed 8 weeks after implantation (figure 7(d)). In contrast, only fibrotic changes were observed in untreated groups, even at 8 weeks (figure 7(a)(b)). Thus, PPL/β-TCP composite appears to be a useful tool for bone engineering.

4. Discussion

CP cements have been used for bone replacement because of their similarity to the mineral composition of bone. However, mechanical properties of CP cements, including Biopex-R and β-TCP, are relatively weak [19]. In the present study, we demonstrated the compressive strength of PPL/β-TCP composite was greater than that of either Biopex-R or β-TCP, and applied PPL/β-TCP composite to bone engineering. Our results indicate PPL was replaced by bone marrow tissue by 8 weeks. Further, PPL/β-TCP composite successfully induced bone formation when applied to the bone marrow space and bone defects. Thus, PPL/β-TCP composite appears to be biocompatible bone cement.

For reconstructive surgery in a clinical setting, such as vertebroplasty, cements are required to bear the axial load. PMMA cement is suitable for application in load-bearing areas because of its excellent mechanical properties. The compressive strength of PMMA is 170 ± 5 MPa [20], which is within the range of cortical bone (90–209 MPa) [21]. However, several concerns exist for clinical use of PMMA cement. First, PMMA produces heat during polymerization [22], which may cause damage to adjacent tissue. Second, PMMA does not bind to bone, allowing for connective tissue ingrowth between PMMA cement and bone, which may cause aseptic loosening. Third, PMMA is non-biodegradable with no

osteoconductivity [4]; consequently, cement wear particles may initiate aseptic osteolysis though biological reactions [23]. Fourth, the stiffness of PMMA cement is stronger than that of adjacent bone, which may cause iatrogenic fractures [1]. Lastly, bone cement implantation syndrome (BCIS) is a significant cause of morbidity and mortality associated with PMMA cement [24]. In spite of these potential pitfalls, PMMA is widely used in clinical practice because alternative materials possessing appropriate mechanical properties are unavailable. The compressive strength of CP cement is similar to cancellous bone, within 2–20 MPa [25, 26]; this is insufficient for patients with osteoporosis as osteoporotic bone loss also affects cortical bone. Importantly, the compressive force of PPL/β-TCP composite tested as high as ~75 MPa, close to the lower limit of cortical bone (90–209 MPa) [21]. Thus, PPL/β-TCP composite represents a potential substitute for PMMA cement for treatment of osteoporotic vertebral compression fractures. Further studies are necessary to confirm load-bearing capacity of composite mixtures.

In the present study, we demonstrated PPL alone is not capable of inducing bone formation; however, PPL may facilitate bone remodeling in combination with β-TCP. Very recently, we demonstrated titanium implant surfaces treated with PPL positively influence osteogenesis in a rabbit model [15]. Further, polyphosphoric acid-treated titanium increases attachment and proliferation of mouse osteoblast-like MC3T3-E1 cells and bone marrow-derived mesenchymal stem cells [27]. Polyphosphates have been found to induce maturation and calcification of bone-related cells by accelerating alkaline phosphatase and osteocalcin gene expression [28]. Surface phosphorylation of other biomaterials also considerably increases cell attachment and proliferation [29]. Thus, phosphorylated functional groups (dihydrogen phosphate groups) of PPL were expected to facilitate bone formation. We demonstrated PPL/β-TCP composite was replaced by bone at 8 weeks; this rapid absorption potentially provides space for new bone formation, assuming bone marrow-derived osteoblasts infiltrate the space and utilize β-TCP from the composite to facilitate bone formation.

We employed β-TCP, not α-TCP, as a calcium phosphate. Despite identical chemical conditions, these isomers differ in structure, density and solubility. α-TCP is designed to change to apatite [30]; whereas, β-TCP turns to brushite, which is soluble in physiological conditions [31]. It takes approximately 2 years for α-TCP to be absorbed by osteoclastic activity [32]. In the present study, gross appearance of α-TCP remained unchanged at 8 weeks, a time when PPL/β-TCP composite was completely absorbed.

We designed PPL based on a well-documented dental adhesive technology. Grafting many functional groups (dihydrogen phosphate groups) to the biodegradable polymer backbone provides the basis for designing and synthesizing PPL. We demonstrated PPL/β-TCP composite adheres to titanium and hydroxyapatite, suggesting PPL/β-TCP composite adheres directly to existing bone around defects via phosphate groups of PPL. However, the adhesive property of PPL/β-TCP composite was not clearly proven *in vivo* - further studies will be necessary to address this point.

5. Conclusion

Newly developed PPL possesses excellent mechanical and adhesive properties when mixed with β-TCP. PPL/β-TCP composite was biocompatible and induced excellent bone formation, even in a load-bearing vertebroplasty model. Together with good manipulation capability caused by water solubility, we believe PPL is a promising biomaterial for bone formation in combination with β-TCP.

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Figure legends

Figure 1. Chemical structure of PPL (a) and NMR spectra of PL and PPL (b).

Figure 2. Shear bond strength of Biopex-R, β-TCP, PMMA cement, PPL and PPL/β-TCP composite to titanium and hydroxyapatite (a) (n=5 for each). The compressive strength of Biopex-R, β-TCP, PPL and PPL/β-TCP composite was measured (b) (n=4 for each). Data are presented as mean ± SEM. **P*<0.05 between the groups.

Figure 3. Mice were intramedullary injected with PPL or PPL/β-TCP composite. (a) At 2, 5 and 8 weeks after injection, mice were sacrificed and femurs were resected (n=3 at each time point). Tissue sections were stained with hematoxylin and eosin (i-vi). Representative sections are shown. Arrows indicate the bone defect. Arrowheads (close-up images of iv and vi) represent bone formation. (b) At 4 weeks after injection, femurs were scanned by micro-CT. Representative images from each group. (c) Bone mineral density was calculated by micro-CT at 4 weeks after injection (n=4 for each). **P*<0.05 between the groups.

Figure 4. Bone defects were made in the middle of the ulna and defects were filled with Biopex-R or PPL/β-TCP composite (a). Rabbits were sacrificed at 4 and 8 weeks after surgery (n=3 for each). Forearms were removed and scanned by μ-CT (b). Representative images are shown. Closed and open arrowheads represent bone defect and formation, respectively. Representative cut sections of defects are shown (c). Arrows indicate Biopex-R. Circle indicates area of bone healing.

Figure 5. Ulnar bone defects were made in rabbits and defects were filled with Biopex-R or PPL/β-TCP composite. Rabbits were sacrificed at 4 (a)(c) and 8 weeks (b)(d) after surgery (n=3 for each). Forearms were resected and sections were stained with hematoxylin and eosin (HE) and safranin O. Representative sections are shown. Arrowheads indicate fibrous tissue

around Biopex-R. Asterisk indicates a fragmented bone ingrowth.

Figure 6. Either Biopex-R or PPL/β-TCP composite was applied to bone defects in pig vertebrae. (a) Pigs were sacrificed 8 weeks after surgery and spines were resected (n=2 for each). Vertebrae were scanned by μ-CT. Representative cut sections and μ-CT images are shown. Arrowheads indicate new bone formation. (b) CT number (HU) was assessed after treatment with PPL/β-TCP composite. Untreated was used as a defect control (3 vertebrae/pig, 2 pigs for each). The CT number of non-treated vertebrae was 1,030 ± 32 (3 vertebrae/pig, 2 pigs for each). $^{**}P<0.0001$ between the groups.

Figure 7. Vertebra defects were filled with PPL/β-TCP composite. Untreated defects were used as controls. At 4 (a)(c) and 8 weeks (b)(d) after surgery (n=2 for each), pigs were sacrificed and vertebrae were resected. Tissue sections were stained with hematoxylin and eosin. Representative histological images are shown. Arrowheads indicate new bone formation. Asterisks indicate existing bone.