Development of β-TCP based Antimicrobial Injectable Bone Grafts

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***Abstract*—Beta-tricalcium phosphate (β-TCP) is a widely utilized biomaterial in bone grafts due to its osteoinductivity, biocompatibility, and biodegradability. Especially, injectable bone grafts that can be locally applied with a special syringe to irregularly shaped bone defect areas may be advantageous. However, its low mechanical strength limits its effectiveness in load-bearing applications. To overcome this problem, the usage of sintered β-TCP, may provide enhanced mechanical strength to the injectable bone grafts since sintering process increases the mechanical strength of β-TCP and improves its structural stability. Although most of the current strategies for bone regeneration provide relatively satisfactory results, the lack of antimicrobial efficacy in bone grafts limits their use. Current clinical treatment strategies usually involve antibiotic therapy with broad-spectrum antibiotics, following extensive surgical debridement. However, these antibiotics cause bacterial resistance and systemic side effects. Antimicrobial peptides (AMPs) are promising antibacterial agents due to their broad-spectrum antibacterial activity, controllable biocompatibility and limited resistance development. KR-12, a cationic AMP derived from human LL-37, shows strong antibacterial activity, low toxicity and promotes osteogenic differentiation in mesenchymal stem cells, making it a promising candidate for topical application in the management of infections. In this study, we designed an injectable bone graft by combining sintered and unsintered β-TCP in various ratios to enhance the mechanical strength of the grafts. To address the lack of antimicrobial efficacy, we functionalized the grafts with the antimicrobial peptide KR-12, known for its broad-spectrum antibacterial activity and low resistance development. The optimal peptide concentration was determined, creating a graft with both improved mechanical strength and effective antibacterial properties. Future studies will assess its antimicrobial effectiveness and impact on osteogenic differentiation using mesenchymal stem cells.**

Keywords—injectable bone graft, β-TCP, antimicrobial peptide.

I. Introduction

Currently, bone grafts are widely utilized for the effective regeneration of bone defects caused by trauma, infection, tumors, or hereditary genetic disorders. Several synthetic biomaterials such as polymers and ceramics have shown effective biomaterials for the treatment of bone defects [1, 2]. Calcium phosphate ceramics, particularly beta-tricalcium phosphate (β-TCP) are favored due to their desired osteoinductivity, biocompatibility and biodegradability that makes them excellent bone graft substitutes [3]. Although bone grafts play a crucial role in the successful regeneration, their effectiveness is limited for the treatment of geometrically irregular shaped bone defects. Hence, injectable bone grafts that can be locally applied to the surgical site via special syringe to irregularly shaped bone defect areas may be advantageous. These injectable grafts are commonly used in specific indications in the spine, osteoporotic fractures, and craniofacial defects [4]. Most injectable bone grafts require a mixing process of a powder with a liquid to obtain a paste-like form within a limited time. However, the time needed for the desired paste-like form causes significant drawbacks from application to application. To address these challenges, in clinical practice, ready-to-use, package-stable bone grafts, also known as putty grafts, which harden immediately after injection are widely utilized [5]. Despite the favorable osteoinductive properties and biodegradability of β-TCP-based injectable bone grafts, their relatively low mechanical strength limits their use in the load-bearing applications [3]. To overcome this problem, the use of β-TCP in granule form that is sintered, may provide enhanced mechanical strength to the injectable bone grafts. Sintering process increases the mechanical strength of β-TCP and improves its structural stability which makes β-TCP more suitable for use in applications where higher compressive strength is required [6]. Additionally, since the sintering process can enhance the bioactivity of β-TCP, integration of bone graft with the surrounding bone tissue is also improved [7].

Although most of the current strategies for bone regeneration provide relatively satisfactory results, the lack of antimicrobial efficacy in bone grafts limits their use. Despite advanced surgical procedures and antibiotic applications, bone infection remains one of the most devastating complications in trauma and orthopedic surgery [8]. These infections, which generally occur in association with open fracture surgery, bone reconstruction surgery, or orthopedic implants, can become chronic if untreated, leading to non-union of the bone and failed implantation. As the infection progresses, it can cause sequestration, sinus and sepsis formation, and life-threatening complications such as amputation [9]. Current clinical treatment strategies usually involve a 4-6 week course of parenteral antibiotic therapy with broad-spectrum antibiotics like vancomycin, ciprofloxacin, and gentamicin, following extensive surgical debridement [10, 11]. However, these antibiotics, which cause systemic side effects such as organ failure, gastrointestinal side effects, and allergic reactions, may have insufficient local efficacy when they fail to reach adequate concentrations at the

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site of infection [12]. As an alternative to systemic antibiotic therapy, local antibiotic treatment is preferred to enable more effective treatment by increasing antibiotic concentrations at the infection site. For this purpose, antibiotic-loaded polymethylmethacrylate (PMMA) based bone grafts have been used as a local antibiotic carrier biomaterial in bone infection treatment in recent years [9]. However, PMMA's low antibiotic release rate, low biocompatibility, non-biodegradable nature requiring additional surgical removal, and potential to cause thermal necrosis limit its clinical use and may lead to antibiotic resistance and biofilm formation due to the low antibiotic concentration it can incorporate [13]. Recently, intensive research has been conducted on functionalizing biodegradable bone grafts with antibiotics to eliminate these disadvantages [13, 14]. Studies have found that grafts containing high concentrations of antibiotics are needed to eradicate bacterial infection and/or biofilm; however, high concentrations of antibiotics can cause osteoblast toxicity [15, 16]. Although the theoretical usage of antibiotic-loaded bone grafts in orthopedic surgery is valuable, their clinical use remains uncertain [14]. For these reasons, there is a significant need for new antibacterial agents that are effective in preventing or treating bone infections in bone defect repair while limiting the development of resistance [17].

Antimicrobial peptides (AMPs), also known as cationic host defense peptides, are promising agents for infection treatment due to their broad-spectrum antibacterial activity, controllable biocompatibility and limited resistance development. Unlike traditional antibiotics, AMPs rarely induce resistance due to their multiple antibacterial activities and attacks on low-affinity targets like bacterial membranes rather than specific molecules [18]. Given the challenges of systemic application due to rapid degradation in the blood and low stability, topical application of AMPs is recommended for trauma and infection treatment [17]. KR-12, a cationic AMP derived from human LL-37 with the sequence KRIVQRIKDFLR (K: Lys; R: Arg; I: Ile; V: Val; Q: Gln, D: Asp; F: Phe, L: Leu), shows strong antibacterial activity against both gram positive and gram negative bacteria, low toxicity and promotes osteogenic differentiation in mesenchymal stem cells, making it a promising candidate for topical application in the management of infections [19, 20].

In this study, we developed a β-TCP based injectable bone grafts with increased mechanical strength were developed by combining sintered β-TCP granules and unsintered β-TCP powder in different ratios. To functionalize developed injectable bone grafts with KR-12 AMP to provide antibacterial activity, FITC labeled KR-12 peptide was conjugated to sintered β-TCP granules at different concentrations and the optimum peptide molarity in the developed putty graft was determined.

II. MATERIALS AND METHODS

A. Production of Injectable Bone Grafts

In the production of β-TCP based injectable putty graft, unsintered β-TCP powder, sintered β-TCP granules and hydroxypropyl methylcellulose (HPMC) were mixed in different ratios. As a control, putty graft was prepared by using only β-TCP powder and HPMC. Briefly, 3% (w/v) HPMC solution was prepared and dissolved with a mechanical mixer at 360 rpm and 80°C for 2 h until completely homogenized. Afterwards, a mixture of 54% β-TCP powder, 43.2% HPMC solution and 2.8% HPMC by mass was kneaded until dough-like form was obtained. The same protocol was followed for the production of other putty grafts by adjusting the β-TCP powder: β-TCP granule: HPMC ratio by mass as follows:

1) 1:0:1 (Control Putty)
2) 0.5:0.5:1
3) 1:0.5:1.5
4) 0:1:1

Finally, injectable putty grafts that had the desired dough-like form were filled into the syringe for future use.

B. Mechanical Characterization of Produced Injectable Bone Grafts

The injectability of the putty grafts which are prepared by adjusting the ratios of β-TCP powder, β-TCP granule and HPMC was observed and evaluated by photographs. Furthermore, to determine the setting-time for the grafts to harden completely, the putty grafts were injected in a volume of 50 µl and the setting-time was determined. To determine the microhardness of the grafts, putty grafts were produced in the form of a 1 cm diameter disk and completely hardened. Afterwards, Vickers microhardness was measured by applying a 100 gf load. The group that gave the most successful results in terms of injectability, setting-time and microhardness was selected for the further experiments.

C. FITC Labeled KR-12 Antimicrobial Peptide Synthesis

KR-12 peptide with a sequence of (KRIVQRIKDFLR) was synthesized on 4-Methylbenzhydrylamine (MBHA) Rink Amide resin with a substitution of 0.67 mmol/g via 9-fluorenylmethoxycarbonyl (Fmoc) chemistry [21]. Briefly, 300 mg of the resin was swollen in dimethylformamide (DMF) for 30 min. Subsequently, 20% 4-methyl piperidine/DMF solution was added to the resin to remove the Fmoc protection and incubated in an orbital shaker for 30 min. To detect the presence of amines, ninhydrin test was performed. Then, the resin was washed 3 times with DMF and 2 equivalents of Fmoc-protected amino acid, 2 equivalents of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate (HBTU) and 4 equivalents of N,N diisopropylethylamine (DIEA) were dissolved in DMF and added onto the resin. The resin was incubated with amino acid solution on the orbital shaker and mixed for 3 hours for the reaction to take place. After 3 hours, a ninhydrin test was performed to determine the successful reaction. Then, the resin was washed 3 times with DMF and a DMF solution containing 20% 4-methyl piperidine will be added onto the resin to remove the Fmoc protection. After the entire sequence was completed by using the same steps, KR-12 peptide was labeled with FITC molecule. During this process, the amine group of the peptide and the succinimidyl ester functional group of the FITC molecule was utilized. Finally, FITC labelled KR-12 peptide was cleaved from the resin by using a TFA-based solution (TFA (Trifluoroacetic Acid): TIPS (triisopropylsilane): H_2O (distilled water)) at a ratio of 95: 2.5: 2.5. After 2 hours of incubation of resin with TFA based

solution, the peptide solution was poured into cold ether and the suspension was centrifuged at 4500 rpm, 4°C for 10 minutes to obtain the peptide pellet. The synthesized peptide was frozen at -80°C and lyophilized to form a powder.

D. Functionalization of β-TCP granule with KR-12 AMP

In the functionalization of putty grafts with KR-12 peptide, FITC-labeled KR-12 peptide chemically conjugated to β-TCP granules in the structure of putty graft containing β-TCP powder: β-TCP granule: HPMC in a ratio of 0.5: 0.5: 1 [22]. Since there is no -COOH or -OH in β-TCP, cold atmospheric plasma treatment was applied to β-TCP granules with 40 kV voltage and 20 min application time in order to functionalize β-TCP. A 3-step chemical protocol was used to ensure conjugation after plasma treatment. As the first step, plasma-treated β-TCP granules were incubated in ethanol solution containing 20 mmol/L APTES for 4 hours at room temperature. Then, ethanol solution was removed and β-TCP granules were washed 3 times with ethanol. Then, APTES-bound β-TCP granules were incubated in MES buffer (pH 6) containing 0.3% (w/v) 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride for 6 hours at room temperature. After 6 hours of incubation, the solution was removed and β-TCP was washed 3 times with MES buffer. In the last step, FITC-labeled KR-12 peptide solutions of different molarities (300 µM, 500 µM, 800 µM, 1 Mm, 1.5 mM and 2 mM) were prepared in PBS and added onto the β-TCP granules. β-TCP granules were incubated with the peptide solutions for 4 hours. After incubation, peptide solutions were removed from β-TCP and the granules were washed 3 times with PBS.

E. Characterization of FITC-KR-12 Conjugated β-TCP Granules

For the characterization of KR-12 conjugated putty grafts, firstly peptide concentration that is conjugated to β-TCP granules were determined. Briefly, peptide conjugated β-TCP granules were dissolved in DMSO and the solution was transferred to a 96-well plate. Fluorescence reading was performed using a multi-plate reader with 520 nm emission and 495 nm excitation. To determine the amount of AMP conjugated to the β-TCP, a calibration curve was created using serial dilutions of FITC using 520 nm emission and 495 nm excitation values, and the amount of conjugated peptide was obtained with the calibration curve. According to the results obtained from the analyses, the peptide concentration into putty graft was also calculated and optimum conjugation concentration was determined.

III. RESULTS AND DISCUSSION

The injectability of the developed grafts was evaluated (Fig 1) by using a putty graft with a 1:0:1 ratio as the control group. The injectability of putty graft prepared with a 0.5:0.5:1 ratio was nearly the same with the control group. However, when the HPMC ratio was increased in the putty graft to a ratio of 1:0.5:1.5, the injectability decreased compared to the other two groups. This reduction in injectability can be associated with the fact that grafts prepared with an increased amount of HPMC had a denser structure. Moreover, a 1:0.5:1 ratio was also evaluated while keeping the HPMC amount constant. However, the desired putty consistency could not be obtained. Therefore, increasing the amount of HPMC in this group was essential. Furthermore, the graft prepared with a 0:1:1 ratio, using only β-TCP granules lost its injectability. Based on these observations, the graft with a 0.5:0.5:1 ratio was considered the most suitable group to provide optimum injectability.

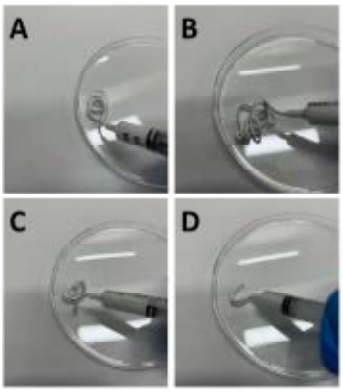


Fig 1. Injectability of developed bone grafts with a β-TCP powder: β-TCP granule: HPMC ratio of A) 1:0:1, B) 0.5:0.5:1, C) 1:0.5:1.5, D) 0:1:1.

In addition to injectability, the setting time required for the hardening of the grafts is a crucial evaluation criteria. The setting time for the control putty graft, prepared with a 1:0:1 ratio, was 64 minutes. The setting-time of the other groups was determined as 69, 88 and 111 minutes for 0.5:0.5:1, 1:0.5.1.5 and 0:1:1, respectively (Table 1). Similar to the injectability results, the setting-time for the graft with a 0.5:0.5:1 ratio was comparable to the control. However, the hardening time of the graft increased undesirably as the HPMC ratio increased. Moreover, the setting-time for the grafts produced using only granules was significantly longer than other groups, which could pose a potential disadvantage in clinical applications. The Vickers microhardness of the developed putty grafts was evaluated (Table 1). According to the results, the microhardness of the control group was measured as 3 HV. The 0.5:0.5:1, 1:0.5:1.5 and 0:1:1 groups were determined as 8.6 HV, 4.2 HV and 12.7 HV, respectively. Studies have shown that the microhardness of human bones is generally above 20 HV, and these values may increase from bone to bone [23]. Considering this, increasing the microhardness of the developed injectable bone grafts has the potential to provide an advantage for the development of a graft that is similar to the bone structure. Furthermore, the results showed that the incorporation of sintered β-TCP to the putty graft increased the microhardness of the grafts compared to the control group. The microhardness of the graft prepared in a 0:1:1 ratio using only granules was the highest among all groups. However, since this group lost its injectability and the setting-time was significantly longer than the other groups, making it unsuitable for clinical use. Considering all the results, the graft prepared with a 0.5:0.5:1 ratio was deemed optimal for bone graft design due to its desired injectability, setting-time and high microhardness compared to control.

In the second step of the study, peptide concentration was optimized by conjugating peptides at different molarities to the granule form β-TCP that will be added into the putty graft. The amount of peptide conjugated to 100 mg β-TCP granule with the

peptide solution prepared at different concentrations was determined using the calibration curve (Table 2). The amount of peptide which is conjugated to β-TCP was increased with increasing concentration of peptide in direct proportion. Considering these results, it was decided to perform the conjugation process using 2 mM peptide concentration for further studies.

TABLE I. ASSESMENT OF INJECTABILITY, SETTING-TIME AND MICROHARDNESS OF BONE GRAFTS

	Ratio of β-TCP powder: β-TCP granule: HPMC			
	1:0:1	***0.5:0.5:1***	***1:0.5:1.5***	***0:1:1***
Injectability	Yes	Yes	Moderate	No
Setting time (minutes)	64	69	88	111
Vickers Microhardness (HV)	3	8.6	4.2	12.7

In the further studies, the antimicrobial tests will be conducted on bone grafts prepared by mixing KR-12 conjugated β-TCP granules at a 0.5:0.5:1 ratio. The antimicrobial effectiveness of the developed bone graft will be evaluated against both gram-positive and gram-negative bacteria. Furthermore, the activity of the developed bone grafts on cell viability and osteogenic differentiation will be evaluated on mesenchymal stem cells.

TABLE II. THE CONJUGATION AMOUNT OF KR-12 TO 100 MG Β-TCP GRANULES WITH DIFFERENT MOLARITIES

AMP Conjugation Amount to β-TCP	
Utilized KR-12 Concentration for Conjugation	***Conjugated Concentration (μM)***
300 μM	57.3
500 μM	150.3
800 μM	187.8
1000 μM	654.4
1500 μM	1046.9
2000 μM	1083.2

IV. CONCLUSION

In this study, novel injectable bone grafts were developed with the combination of sintered and unsintered β-TCP in different ratios with improved mechanical properties. For the further studies, bone graft with a 0.5:0.5:1 ratio will be used due to its optimum properties with improved microhardness. Furthermore, the conjugation of KR-12 peptide to β-TCP granules, which is the first step in the design of injectable bone grafts, was investigated. In further studies, conjugation will be performed at 2000 μM peptide concentration and both the antimicrobial activity and the osteogenic differentiation capacity of the developed injectable bone grafts will be investigated.

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