Development of Antimicrobial Peptide Conjugated β-TCP Discs For Prevention and Treatment Of Bone Infections

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***Abstract*- Osteomyelitis treatment remains one of the most challenging problem despite the advancements in medical technology. While acute infections can be treated with antibiotics, chronic cases requires surgical interventions. With the increase and uncontrolled use in antibiotics, antimicrobial resistance have become a worldwide issue. The increasing concurrency of antibiotic-resistant organisms leads scientists to search for novel treatment methods in the management of osteomyelitis. Recent advances focus on antibacterial biomaterial development, including implants, hydrogels, bone cement and scaffolds. Novel approaches include, antifouling coatings, biofilm disrupting enzymes, bacteriophage-loaded hydrogels, and antimicrobial peptides (AMPs). AMPs show promise in the treatment of bone infections due to its activity against wide range of bacteria without causing damage to human osteoblasts. β-TCP is one of the most commonly used bone graft materials. Although the effectiveness of β-TCP in bone regeneration is known, its lack of antimicrobial activity limits its use. Therefore, a novel bone graft was developed by combining AMP to β-TCP discs for the prevention and treatment of bone infections. Herein, TetraF2W-RR AMP with proven high efficacy against methicillin-resistant Staphylococcus Aureus (MRSA) is conjugated to β-TCP discs. βTCP discs were exposed to cold atmospheric plasma to functionalize the surface with –OH group and the APTES in ethanol is used for silanization. Afterwards, TetraF2W-RR peptide is conjugated to β-TCP discs after EDC activation. AMP conjugated β-TCP samples were characterized with Raman spectroscopy. The efficacy of developed biomaterials were investigated on MRSA strains using colony counting assay. Novel β-TCP/AMP bone grafts provided 2 log bacterial death. In the future experiments, the anti-biofilm activity, cytotoxicity and osteogenic differentiation of developed bone grafts will be investigated.**

Keywords—Osteomyelitis; Antimicrobial Peptides; Cold Atmospheric Plasma

I. INTRODUCTION

Various synthetic materials, including metals, polymers, and ceramics, are used in the treatment of bone defects. The most commonly used materials in bone grafts are hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP). Since HA implants have a much more crystalline structure than bone minerals, they are chemically more stable and do not undergo biodegradation after implantation. This leads to various bone defects and fractures in the long term [1]. β-TCP is a biocompatible ceramic that provides a suitable environment for cell adhesion and proliferation due to its porous structure [2]. Unlike HA implants, β-TCP, which is known to have high osteotransductive and osteoinductive potential, is often preferred in bone grafts since it is easily absorbed by osteoclasts and successfully supports bone formation [1]. Although the effectiveness of β-TCP on bone regeneration is known, the limited antimicrobial activity of βTCP limits its use [3].

The reccurent nature and high morbidity of chronic bone infections, escpecially implant-associated osteomyelitis, pose signiciant challenges in orthopedic medicine [4, 5]. Traditional treatments of osteomyelitis have evolved with time, focusing on antibiotic therapy and surgical interventions. Traditionally, systemic antibiotic therapy with broad-spectrum antibiotics such as vancomycin, ciprofloxacin, and gentamicin is applied to eliminate bacterial infection in the treatment of bone infections [6]. However, systemic application of these antibiotics causes systemic side effects. Furthermore, the desired antibacterial activity cannot be achieved since their concentration at the infection site remains locally low [7]. For this reason, the local application of antibiotics to increase their concentration at the infection site has become an important research topic. However, the management of bone infections using antibiotics is facing an increasing challenge which is antimicrobial resistance (AMR). AMR occurs when microorganisms, including bacteria, viruses, parasites, and fungi adapt to the presence of drugs over time and develop a resistance mechanism against drugs. This situation leads to problems such as prolonged hospital stays, increased care costs, and ineffective treatment during the prevention and treatment phase of infection [8]. Therefore, the use of antimicrobial agents that provide successful eradication of bacteria without causing antimicrobial resistance instead of antibiotics has become very important [9].

Antimicrobial peptides are among the most frequently investigated antimicrobial agents in the treatment of antibioticresistant infections, which are increasing in number due to their strong activity against multidrug-resistant microorganisms [10, 11]. Therefore, the conjugation of AMPs on biomaterials is considered to be one of the most suitable

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approaches for the treatment of infections caused by multidrug-resistant pathogens [12]. TetraF2WRR peptide with the WWWLRRIW sequence is known as one of the important antimicrobial peptides with strong antimicrobial activity on gram-positive bacteria. Especially the WWW motif at the Nterminal targets the bacterial membrane and plays an important role in effectively killing the bacteria with no risk of AMR occurence [13, 14]. Therefore it is considered a promising therapeutic option as an alternative to antibiotics in the treatment of various infections. In a study TetraF2W-RR conjugated with Ce6, which is a photosynthesizer, and the antimicrobial activity on clinic isolate MRSA in vitro is investigated. Ce6-AMP combination exhibited excellent activity agains MRSA [14]. Furthermore, the peptide can be immobilized on biomaterials for prevention of biofilm formation and it has the same antimicrobial effect both in free and immobilized forms [13].

This study aimed to develop a novel β-TCP based antimicrobial bone graft by conjugating TetraF2W-RR AMP to β-TCP discs. In this regard, studies were conducted to examine the effects of immobilizing TetraF2W-RR on β-TCP and the effect of this modification on microbial growth of clinical isolate MRSA were investigated. Briefly, TetraF2WRR peptide was synthesized by solid-phase peptide synthesis. Afterwards, β-TCP discs were exposed to cold atmospheric plasma (CAP) for 20 minutes. The optimization of the plasma duration was determined by analyzing the peaks in the Raman Spectroscopy. The conjugation of AMP on β-TCP discs was conducted using APTES silanization followed by EDC activation after CAP treatment. The antimicrobial effect of developed β-TCP discs was investigated on MRSA using colony counting assay.

II. Material And Methods

A. Solid Phase Peptide Synthesis

Peptide synthesis materials were acquired form AAPPTEC (Louisville, KY, USA). Antimicrobial peptide, TetraF2W-RR was synthesized using the fluorenylmethoxycarbonyl (Fmoc) chemistry. 800mg 4- methylbenzhydrylamine (MBHA) resin (0.53 mmol/g loading capacity) was left in N, N-dimethylformamide (DMF) for 30 minutes. Aminoacid coupling was conducted using Fmoc protected amino acids (2 equivalent), N, Ndiisopropylethylamine (DIEA; 4 equivalent), and 2-(1Hbenzotriazol-1-yl)- 1,1,3,3- tetramethyluronium hexafluorophosphate (HBTU; 2 equivalent) in DMF. The amino acid solution was left to stir with resins for 4-6 hours. For the deprotection of Fmoc-protecting groups resin was left in 20% piperidine in DMF solution for 30 minutes. After every aminoacid coupling and deprotection step, Ninhydrin (Kaiser) test was realized to detect unreacted amine presence. Coupling and deprotection steps are repeated until all the amino acids are coupled to the peptide sequence. After all the sequence is completed, the resin was left in cleavage solution consist of trifuoroacetic acid (TFA) (95% (v/v)), triisopropylsilane (TIPS) (2.5% (v/v)), deionized water (2.5% (v/v)) for 2 hours to seperate resin from the peptide sequence. Then the solution was poured into cold ether and the product was centrifuged at 4500 rpm for 10 minutes with 3 repetitions. The remaning ether in the precipitate was removed by vacuum evaporator and the solid part was freeze dryed [15].

B. Kinetic Growth Analysis of Planktonic Form

Planktonic form of MRSA was used to evaluate the antimicrobial activity of the AMP. Frozen MRSA strain was suspended in tryptic soy broth (TSB), incubated at 37°C in a shaker incubator overnight and then streaked on tryptic soy agar (TSA) plates. Before the experiment, one colony was picked from agar plates, suspended in TSB and incubated at 37°C overnight. 10^8 CFU/ml concentration was adjusted by measuring the optical density at 600 nm using spectrophotometer (PG Instruments Limited, Leicestershire, UK). Effective peptide concentrations were found from the literature and peptide solution was prepared accordingly. Bacteria suspension was diluted to 10^5 CFU ml^{-1} and mixed with the prepared peptide solution. At 600 nm every 20 minutes for 18 hours the optical density was obtained in multiplate reader.

C. Cold Atmospheric Plasma Application

β-TCP discs were plasma treated with a microsecond pulsed high voltage source. This source was linked to a 50 mm by 75 mm dielectric barrier discharge electrode. Samples were plasma treated for twenty minutes at 40 kV. 20 minutes of plasma application was optimized with Raman Spectroscopy measurements.

D. Functionalization of β-TCP Discs with Antimicrobial Peptide

Functionalization of β-TCP discs with AMP is conducted by chemically conjugating the TetraF2W-RR to β-TCP discs. β-TCP does not include –OH or –COOH in its structure. In order to add functional groups to β-TCP cold atmospheric plasma was applied for 20 minutes with 40kV voltage. Plasma treated β-TCP discs were left to incubate in 20 mmol/L APTES containing ethanol solution for 4 hours at room temperature. After that the β-TCP discs were rinsed with ethanol 3 times and APTES- β-TCP discs were obtained. Then, the samples were incubated in MES buffer with pH 6 containing 0.3% (w/v) 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride. After 6 hours of incubation, samples were rinsed with MES buffer 3 times. Finally, 1mM peptide solution in PBS is added to β-TCP discs amd left for conjugation at room temperature for 4 hours. After that, before the experiments the samples were rinsed with PBS 3 times [16].

E. Colony Counting Assay

The organism culture that was suspended at 10^8 CFU/ml were diluted to 10^5 CFU ml^{-1} with serial dilutions. 100ul were transferred onto the peptide conjugated β-TCP discs and microorganisms were left on β-TCP samples. After 24 hours the solutions on the β-TCP samples were diluted to 100 CFU/ml and each dilution was spread on agar plates. After 24 hours of incubation at 37°C and viable colonies were counted.

F. Evaluation and Statistical Analysis of the Results

For all statistical analyses, two-way ANOVA and Student-Newman Keuls method were used. Significant differences between groups were determined at p values of at least 0.05 (* $p < 0.001$).

III. Results And Discussion

Kinetic growth of MRSA with different concentrations of TetraF2W-RR AMP is given in Fig.1. The measured absorbance value was increased as expected, with the increase in the number of microorganism with time. Based on this information, it can be seen that the duration MRSA entered the log phase (growth phase) has increased with the increase in peptide concentration. 100 µM and 200 µM showed bacteriostatic effect, which means the AMP suppressed the MRSA growth for several hours more. After 300 µM peptide concentration there was not any bacterial growth seen. Considering these, TetraF2W-RR provided bactericidal effect at concentrations higher than 300 µM.

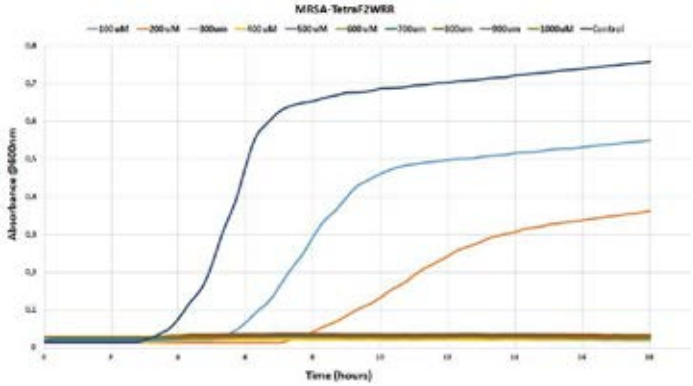


Fig. 1. Growth kinetics of 10^5 CFU ml^{-1} MRSA in different concentrations of TetraF2W-RR peptide.

Raman spectra was utilized for the characterization of TetraF2W-RR AMP conjugated β-TCP discs. After 20 minutes of plasma application, a significant extension was observed in the peaks around 1000 cm^{-1} in the Raman spectrum of β-TCP, indicating $PO4^{3-}$. In the β-TCP/AMP spectrum, a significant shortening was observed in the peaks around 1000 cm^{-1}, especially compared to the spectrum of βTCP/plasma. Moreover, new peaks were formed around 500 cm^{-1}. This can be attributed to the fact that the newly formed peaks may presence of various amino acids. Especially, Raman signals of non-aromatic amino acids are observed between 500 and 1700 cm^{-1}. Moreover, new peaks formed between 500 cm^{-1} and 1000 cm^{-1} indicate C-O-C and C-H stretches due to presence of peptide [17]. These results confirmed the successful β-TCP modification by plasma application and conjugation of the TetraF2W-RR peptide.

The antimicrobial activity of AMP conjugated β-TCP discs were investigated after 24-h contact in 10^5 CFU/ml MRSA. As depicted in Fig 2. β-TCP by itself did not provide any antibacterial effect and there was not any significant difference compared to control in the MRSA viability on β-TCP discs.

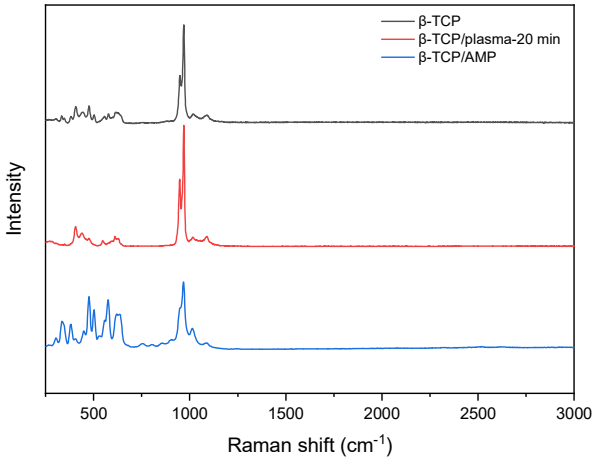


Fig 2. Raman spectra for β-TCP, β-TCP after CAP treatment for 20 minutes (β-TCP/plasma-20 min) and TetraF2W-RR peptide conjugated β-TCP (β-TCP/ TetraF2W-RR).

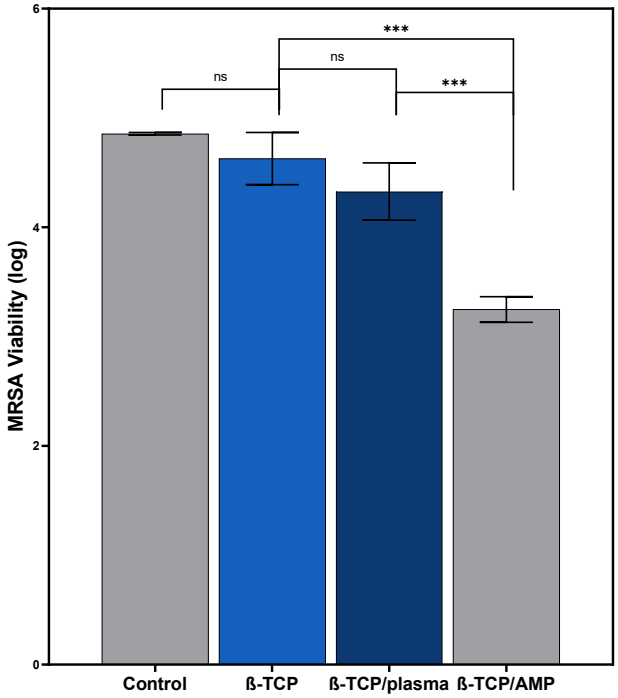


Fig. 3. Colony counting results of 10^5 CFU/ml MRSA, after 24-hour incubation in PBS (Control), on β-TCP, on plasma applied β-TCP (β-TCP /Plasma), and the conjugate of TetraF2W-RR (β-TCP /Peptide). Error bars represent mean ± SE (n = 3)

Since the plasma application by itself can cause antibacterial effect in several situations [18], MRSA viability was also investigated on plasma applied β-TCP discs. However, there were no significant changes in the bacterial viability compared to control MRSA and β-TCP. Moreover, TetraF2W-RR conjugated β-TCP discs showed bactericidal effect with providing almost 2 logs bacterial death. These results demonstrated the strong antimicrobial activity of the

developed β-TCP disks against MRSA while also confirming that this antimicrobial activity is solely due to the AMP.

IV. Conclusion

Herein, a novel β-TCP based antimicrobial bone graft was developed and its activity against MRSA was shown. The newly developed bone graft caused 2 log bacterial death showing effective eradication on MRSA. β-TCP/AMP bone grafts has a tremendous potential for the treatment of osteomyelitis in clinical applications due to its strong antibacterial activity. In the future studies, the antibacterial activity of bone grafts will be evaluated on *E. Coli* which is a gram-negative bacteria. Furthermore, the anti-biofilm activity and osteogenic differentiation capacity of β-TCP/AMP will be investigated.

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