PROJECT COMPLETION REPORT

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Title of the project:
Anti-microbial peptide (LL37) loaded Multifunctional 3D collagen scaffold for vascularized bone tissue regeneration

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1. Objectives as stated in the project proposal:
   - To develop a functional 3D scaffold with antimicrobial activity and improved bioactivity that enhances not only osteogenesis and bone regeneration but also neovascularization in bone defect model.
   - A 3D collagen scaffold coated with LL37-loaded HAp layer will be fabricated by using the Oyane's biomimetic process.
   - Evaluation of biocompatibility of 3D scaffold by analyzing cell-material interactions in both *in vitro* and *in vivo* systems.

Methodology:

- **Fabrication of 3D collagen scaffold coated with LL37-loaded HAp layer**

3D collagen sponges will be coated with LL37-loaded HAp layer using precursor assisted biomimetic process developed by Oyane and co-workers. Briefly, collagen-based 3D sponge will be subjected to oxygen plasma and alternate immersion treatments for pre coating calcium phosphate on the sponge surface and treated sponge will be immersed in supersaturated calcium phosphate solution.

- **Surface morphology and chemical composition**

The outer and inner surfaces of the fabricated sponges were characterized before and after the biomimetic coating process by various analytical techniques like scanning electron microscopy (SEM) and for chemical analysis by energy-dispersive X-ray spectrometry (EDX) and X-ray photoelectron spectroscopy (XPS).

- ***In vitro* studies**

In vitro studies were carried out using evaluated using Human Osteosarcoma (HOS) cell line.

- ***In vivo* osteogenesis**

The bone implantation study was planned for a period of 4 weeks and 12 weeks. The scaffold will be implanted in defected site of rabbit's bone. Bone formation on/in the scaffold (including measurement of new bone formation and osteointegration) was studied by histological procedures. Pre and post operative care was done accordingly as per ISO 10993-2.

Fabrication of 3D collagen scaffold coated with LL37-loaded HAp layer

Successfully prepared the mineralized collagen sponges using the biomimetic process. The precursor-assisted biomimetic process is composed of three steps: (1) plasma treatment; (2) an alternate dipping treatment for Calcium Phosphate (CaP) precoating; and (3) immersion in a Supersaturated CaP solution for apatite coating. Plasma etching has been used for the surface treatment of collagen sponges *(6mm x 2mm x 1mm)* for enhancing the surface wettability. Plasma-treated sponges were then coated with CaP by individually immersing in calcium and phosphate solutions, using a vacuum degassing system termed as alternate immersion treatment. The precoated sponges were then immersed in supersaturated CaP solution for 48hrs.The standardized protocol for the mineralized collagen scaffold preparation is shown in Fig.1

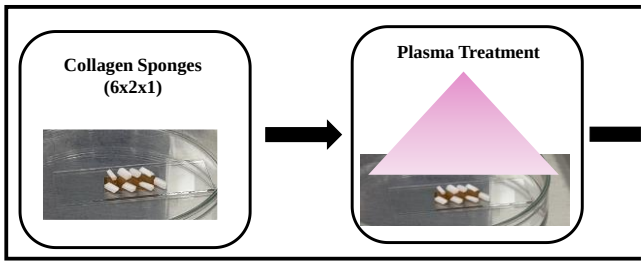

Fig. 1: Standardized protocol for the fabrication of mineralized collagen scaffolds

Surface morphology and chemical composition

EDX analysis showed that all the deposition contained an amount of calcium (Ca) and phosphate(P) when compared to the untreated sponges. XRD analysis also revealed the presence of apatite coating on the collagen sponges. The morphological observation carried out using SEM revealed the uniform coating of apatite on the outer as well as the inner surfaces). In summary, we fabricated antimicrobial LL-37 peptide loaded multifunctional 3D collagen scaffold. We initiated the *in* vitro and *in* vivo studies of the fabricated collagen scaffold.

After application of the biomimetic process, characteristic apatite peaks ((002) and (211) at 2θ = 25.88 and 31.77°, respectively) appeared, while the broad halo due to the amorphous phase disappeared for all of the sponges. These results demonstrated the formation of apatite coatings on the collagen sponges. The apatite-coated sponges were slightly hardened but seemed to be similar in size and shape to the untreated sponges.

***In vitro* studies: Cell-material interaction studies & Cytotoxicity**

Direct contact *in vitro* cytotoxicity test-Qualitative evaluation by phase contrast microscopy

HOS cells were seeded at a concentration of 5 x 10^4 cells /well and LL-37 loaded collagen scaffold with HaP layer was carefully kept at the center of the well with subconfluent monolayer to cover approximately one tenth of cell surface. The plate was

incubated at 37°C under 5 % CO_2. The cytotoxic effects were qualitatively assessed as per ISO: 10993-5: 8.5.1, using an inverted microscope (Leica) after 24h incubation. The cells were qualitatively assessed by microscopical examination for morphological changes, vacuolization, and cell detachment, lysis of cell and membrane integrity. The cells in direct contact with high density polyethylene and organotin stabilized polyvinyl chloride (o-PVC) served as negative and positive control respectively. The HOS cells did not show any morphological alterations after 24h direct contact incubation with material. Cell membrane integrity is also retained with no cell lysis, vacuolization and cell detachment when compared with the negative and positive control.

***In vitro* Angiogenesis**

The *in vitro* angiogenic activity of the 3D scaffold loaded with antimicrobial peptide LL-37 was assessed by an angiogenesis kit. Briefly, Human umbilical vein endothelial cells (HUVEC) were cultured and incubated with HaP coated 3D collagen scaffolds with varying concentration of antimicrobial peptide LL-37. The results showing the tubule formation in HUVEC cells suggests that the fabricated HaP coated 3D collagen scaffold loaded with antimicrobial peptide LL-37 stimulates the angiogenesis which is indispensable for successful bone regeneration. Hence the fabricated material can be an ideal candidate for bone regeneration.

***In vitro* Osteogenesis using ALP assay**

The ALP staining and quantitative assay of were performed with alkaline phosphatase kit (Abcam) after culture for 3 and 7 days, respectively. Alkaline phosphatase (ALP) activity was assayed using colorimetric ALP kit (Abcam, U.K). Briefly, HaP coated 3D collagen scaffolds were treated with varying concentration of antimicrobial peptide LL-37andwere grown with HOS cells. The cell culture plates were then incubated at 37 °C with 5% CO_2 for 3 and 7days. As per manufacture protocol, cells were harvested and incubated with 50 µL of *p*-nitrophenyl phosphate (5mM) solution at room temperature for 1 h in the dark. At the end of the incubation, enzyme activity was stopped by adding 20 µL of stop solution. The alkaline phosphatase activity was measured at 405 nm.

Extracellular mineral (Ca^{+2}) deposition assay

Mineralized calcium deposition was assessed by Alizarin Red S staining. Calcium uptake by HOS cells were monitored by incubating cells with scaffold and LL37 for 3,7and 14 days. After the incubation period, the treated cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 20 min. The fixed cells were stained with freshly prepared alizarin red (2% aqueous, pH 4.2) and washed with distilled water prior to microscopic observation. Intensity of the stain uptake was used to assess the calcium accumulation and compared with the untreated control using inverted microscope.

Cell Viability Studies

HOS cells at a concentration of5 x 10^4 cells/well were seeded on to LL-37 loaded HaP coated 3D collagen scaffolds. Cells were incubated for 3, 7 and 14 days at 37°C and calcein AM/PI staining was done to confirm viability of cells in the scaffolds. Briefly, calcein AM (1:1000 from 1.005mM stock) and PI (1:200 from 1.5mM stock) prepared in serum free DMEM was added to materials washed 3 times in DPBS and incubated for 30 minutes. Images were taken using confocal microscopy. Further confirmed the viability

of the HOS cells by live dead assay using calcein AM and PI.The results suggested the effective attachment and proliferation of HOS cells on to the fabricated scaffold.

The mineralized collagen-based sponges possess a 3D porous structure with a mineralized surface appropriate for cell adhesion and in-growth, and hence, have potential use as bone tissue engineering scaffolds. Once the mineralized collagen-based sponges are implanted in the bone defect, they should bond to the surrounding bone tissue due to the biocompatibility and osteoconductivity of the apatite coating.

In vivo studies:

New Zealand white rabbits, both male and female with body weight not less than 2kg is selected for the study. The bone implantation study was planned for a period of 4 weeks and 12 weeks.

Hematology:

Blood samples are collected from both 4weeks (n=3) and 12 weeks animals (n=3) and examined for the following parameters: white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin concentration (Hb), platelets (PLT)

Biochemical analysis:

Blood was collected from both 4weeks (n=3) and 12 weeks animals (n=3) and serum separated by centrifugation at 3000 rpm for 5 min,following parameters alanine amino transferase(ALT), aspartate amino transferase(AST), alkaline phosphatase (ALP), triglycerides, albumin, total protein, total bilirubin, glucose, cholesterol,Creatinine and BUN (Aggappe Diagnostic Ltd, Kochi, Kerala, India) were estimated using an automatedbiochemical analyzer (ERBA XL 300, Germany) andas per the manufacturer's instructions.

Histopathology

The test and control implant materials along with the femur bone were collected. The sites of implantations were macroscopically examined for any evidence of tissue reaction. Both the test and control implanted materials along with femur bones were fixed, processed and embeded in methyl methacrylate (MMA). After polymerization, Thick sections were cut using linear precision saw microtome (ACCUTOME 100 Struers, Denmark). The sections were stuck to a glass slide, ground and surface was polished using a variable speed grinder polisher (ECOMET 3000, Buehler,Germany).The tissue sections were placed on a glass slide polished using a variable speed grinder polisher (ECOMET 3000, Buehler,Germany) and is stained for histopathological evaluation with hot stevenel, blue. The stained slides were observed under trinocular transmitted light microscope (Nikon Ni Eclipse). Histoimages of bone formation after implantation of scaffold (4 weeks and 12 weeks)

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis were done using one-way ANOVA followed by Tukey's post hoc test. The p values ($p< 0.01$, $p < 0.05$) are considered as significant.

Conclusions

Successfully fabricated 3D collagen scaffold coated with LL37-loaded HAp layer and confirmed the surface morphology using various analytical techniques. The fabricated 3D collagen scaffold exhibited enhanced osteogenic ALP activity as well as excellent angiogenesis properties. *In vivo* the bone implantation study on New Zealand white rabbit

for a period of 4 weeks and 12 weeks showed bone formation, HaP coated 3D collagen scaffold with loaded with antimicrobial LL-37 induce osteogenesis. The fabricated functional 3D collagen scaffold with sustained release of LL37 in the vicinity of porous surfaces of the scaffold will prevent bacterial infection and enhance bone regeneration and neovascularization. The 3D collagen scaffold can be used as bone regenerative material in bone defect areas.