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Preparation And Characterisation of Tcp Incorporated In Gelatin Scaffold

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Abstract
Aim- In this study, we suggest a new biocomposite scaffold composed of gelatin/α-TCP (tricalcium phosphate)/(GTS) which has enhanced mechanical strength and high level of cellular activity. Introduction- To fabricate gelatinscaffold, a temperature-controlled 3D printing process was used and appropriate printing conditions were selected based on rheological data and to show the feasibility as a biomedical scaffold for bone tissue regeneration. Materials and methods- The various physical and biological results, using MG63 (osteoblast-like cells), of the gelatin scaffold were compared with those of a pure gelatin (G) and gelatin/α-TCP (GT) composite scaffold. gelatin scaffolds showed enhanced mechanical properties in dry and wet state compared to those of the G and GT scaffolds. Also, significantly high cell-proliferation and differentiation of MG63 cells were observed in the gelatin scaffold. Results Therefore, the gelatin composite scaffold will be one of highly potential biomaterials to be used in bone regeneration.

Keywords: β-TCPGelatinChitosanTissue engineering scaffoldMesenchymal stem cell

Introduction

This study, we suggest a new biocomposite scaffold composed of gelatin/α-TCP (tricalcium phosphate)/(GTS) which has enhanced mechanical strength and high level of cellular activity. To fabricate gelatin scaffold, a temperature-controlled 3D printing process was used and appropriate printing conditions were selected based on rheological data. To show the feasibility as a biomedical scaffold for bone tissue regeneration, the various physical and biological results, using MG63 (osteoblast-like cells), of the gelatin scaffold were compared with those of a pure gelatin (G) and gelatin/α-TCP (GT) composite scaffold. gelatin scaffolds showed enhanced mechanical properties in dry and wet state compared to those of the G and GT scaffolds. Also, significantly high cell-proliferation and differentiation of MG63 cells were observed in the gelatin scaffold. Therefore, the gelatin composite scaffold will be one of highly potential biomaterials to be used in bone regeneration.

Calcium phosphate (CaP) ceramic materials have been used traditionally in research into bone regeneration and clinical repair of bone defects because of their favorable biocompatibility and osteoconductivity. However, the use of CaP ceramic materials alone is limited because of their brittleness and low plasticity. To overcome these shortcomings, polymer materials have been introduced to form composite scaffolds to improve bone defect repair efficiency and clinical applicability of CaP materials.
A variety of composite scaffolds combining CaP materials and natural or synthetic polymers have been produced by different preparation technologies. Among them, the electrospinning technique has received increasing attention in regenerative medicine because of its attractive features, such as producing ultrafine fibers that mimic physically the natural bone extracellular matrices (ECM) at the nanoscale and the surface morphology, architecture, and performance of these fibers can be modulated by modifying the composition or content of the components. Thus, in the field of bone tissue engineering, it is a rational strategy to develop composite scaffolds with nanofibrous structures to recapitalize the extracellular matrix of bone.

In recent years, electrospun CaP/polymer nanofibrous composites have been recognized as beneficial for the attachment, proliferation, and osteogenic differentiation of osteoblasts, as well as improving the efficiency of bone defect repair . However, the mechanism behind the supportive function of these scaffolds is poorly understood. Recently, reported that nanofibrous hydroxyapatite/chitosan (nHAp/CTS) scaffolds could induce osteogenesis of bone marrow mesenchymal stem cells (BMSCs) through the activation of the bone morphogenetic protein (BMP)/Smad pathway. However, for

biodegradable composite materials containing CaP ceramics, understanding how calcium ions released from these nanofibers microenvironment influence the osteogenic differentiation of MSCs in situ is of crucial importance for optimizing the design of scaffold materials for bone regeneration applications. Extracellular calcium ions are important to enhance the proliferation and phenotype expression of osteoblast cells. Previous reports showed that the effect of calcium ions on the osteogenic differentiation of osteoblast-like cells MC3T3-E1 or human adipose-derived stem cells is concentration-dependent.

Previously, we successfully prepared gelatin/β-TCP composite nanofibers with different contents of β-TCP nanoparticles using the electrospinning technique. The results demonstrated that attachment, spreading, proliferation, and differentiation of human osteosarcoma MG-63 cells increased with increasing content of β-TCP nanoparticles and continuous release of Ca2+ into the medium. In addition, composite nanofibers with a high content of β-TCP led to significant bone formation compared with that of the pure electrospun gelatin scaffolds. However, how these composite nanofibers promote the osteogenic differentiation of BMSCs is largely unknown.

The objective of the present work was to analyze the effect of electrospun gelatin/β-TCP composite nanofibers on the osteogenic differentiation of BMSCs and examine the underlying mechanism in vitro and in vivo. Initially, we assessed the cell attachment, proliferation, and spreading and alkaline phosphatase (ALP) activity of rat BMSCs on gelatin/β-TCP compared with pure gelatin nanofibers. We then detected mRNA levels of osteogenic specific genes and calcium-sensing receptor (CaSR) as a calcium-signaling molecule. Subsequently, we investigated the efficacy of gelatin/β-TCP to induce new bone regeneration and related CaSR expression by surgically creating a critical-sized calvarial defects model

Material and methods

Preparation of multi-sized porous β-TCP scaffold and gelatin coating The β-TCP scaffold was fabricated using template coating and freeze drying methods. The β-TCP slurry was made by dispersing the nano β-TCP powders (OssGen Co., Daegu, South Korea) into distilled water. The organic additives (5% polyvinyl alcohol, 1% methyl cellulose, 5% ammonium polyacrylate dispersant, and 5% *N*, *N*-dimethylformamide drying agent) were added to the slurry to improve the sintering force and to stabilize the scaffold structure. The polyurethane sponges used as template were coated with slurry and dried at room temperature or using the freeze drying method for 12 h, and the β-TCP scaffold was sintered at 1,250°C for 3 h. After the first coating, the micro-sized pore on the scaffold surface was fabricated by needle. The β-TCP scaffold was coated again with slurry and resintered. The final β-TCP scaffold size was 5 × 5 × 5 mm.

The 3% gelatin powder from the bovine skin was melted in distilled water at 45°C. After cross-linking with 0.2% glutaraldehyde, the gelatin was coated on the β-TCP scaffold through the dip-coating method at vacuum environment. Compressed air was blown into the β-TCP scaffold to remove the residual gelatin slurry. The gelatin-coated β-TCP scaffold was dried at 40°C in a vacuum drying oven for removal of the glutaraldehyde.

Characterization of the β-TCP scaffold The surface morphologies of the sintered and gelatin-coated β-TCP scaffold were showed by a field emission scanning electron microscope [FE-SEM] (S-800; Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV. The detailed porosity and thickness of the structure were observed with micro-CT (Skyscan 1076; Skyscan Co., Antwerp, Belgium). The resolution was set at 9 μm, rotation step was 0.6° and rotation angle was 180°.

The compressive strength was measured by a universal testing machine (3366, Instron® Co. Ltd. Norwood, MA, USA) at a crosshead speed of 1.0 mm/min. The compressive strength was calculated from the maximum load by the following equation:

The *S* is the compressive strength (in megapascals), *F* is the maximum compressive load (in newton), and *A* is the surface area of the β-TCP scaffold perpendicular to the load axis (in square millimeters).

Biological evaluation

The biological properties were measured by cell proliferation and differentiation. The mouse osteoblast cell, MC3T3-E1 cell, (ATCC, Rockville, MD, U.S.A.) was used for *in vitro* tests. The cells (1×10^5 cells/100 μl) were seeded on each scaffold for 1, 3, 7, and 14 days in a 37°C, 5% CO_2 incubator. The cell viability was measured by the Cell Counting Kit-8 [CCK-8] (Dojindo Laboratories, Kumamoto, Japan). The tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), was reduced by the dehydrogenases in the cells to show an orange-colored product (formazan). The absorbance was read at 450 nm with an ELISA reader (Benchmark Plus, Hercules, CA, USA).

The cell differentiation was measured by measuring the level of alkaline phosphatase [ALP] activity using the

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Sensolyte®pNPP ALP Assay Kit (Anaspec, Inc., Fremont, CA, USA). The cells were lysed by Triton X-100 (Anaspec, Inc., Fremont, CA, USA) into the kit and reacted with the working solution. The final solution shows a yellow-colored product. The absorbance was measured at 405 nm.

Particle size and phase analysis of β-TCP powder

The XRD pattern of β-TCP powders matched the JCPDS card no. 09-0169, with two intense diffraction peaks at 27.9 and 31.2 corresponding to the (2 1 4) and (0210) crystal planes, respectively . Only characteristic peaks of pure β-TCP could be found in , suggesting that the synthesized powder was phase pure β-TCP with hardly presence of any other secondary phase. The XRD peaks of synthesized β-TCP correlated very well with that of commercially available β-TCP as in SEM examination clearly revealed an average particle size of spherical β-TCP particle varying between 70–100 nm, which is corroborated well with the particle size data obtained from DLS measurement Ca/P ratio of as synthesized β-TCP powder was 1.5, as determined using EDX analysis XRD analysis of TCP scaffold

The X-ray diffraction patterns of pure chitosan, gelatin, β-TCP, and GCT30 nanocomposite scaffold. Chitosan was identified with a characteristic peak at 2θ = 19.5. Gelatin showed a large amorphous hump between 2θ = 20–25 . In the GCT30 scaffold, the chitosan and gelatin peaks were subdued as compared to much highly crystalline β-TCP peaks. The less crystalline nature of GCT30 composite scaffold as compared to β-TCP nanoparticle was due to the presence of predominantly amorphous gelatin-chitosan phase in the scaffold

Biodegradation behaviour

As tissue engineering aims at regeneration of new tissues, the scaffold has to be degraded with the formation of new bone tissues. The degradation behavior of biomaterials in the physiological environment plays an important role in the engineering process of a new tissue. In our work, the *in vitro biodegradation* performance of GCT scaffolds in PBS was investigated. The degradation procedure involves hydrolysis of gelatin and enzymatic degradation of chitosan. The lysozyme present in internal body fluids can hydrolyse the *β*-1, 4*N*-acetyl-glucosamine groups of chitosan macromolecule . The large quantity of hydrophilic amino and carboxyl groups exist in gelatin, makes it degrade quickly. The scaffold degradation provided a progressive weight loss with a degradation rate almost constant over time, up to day 24. shows the effect of β-TCP addition on GCT scaffold after *in-vitro* degradation in PBS at 37 °C for 4 weeks. The degradation percentage dramatically decreased with addition β-TCP up to 30 wt%.

It is reasonable to think that the strong interaction between gelatin macromolecular chains and β-TCP consumed some hydrophilic groups and decreased the solvent uptake, which protects the macromolecules from hydrolyzing. On the other hand, the increase in bond strength in the polymer network required additional solvation energy to disrupt electrostatic interaction between β-TCP and gelatin, chitosan and caused a decrease in degradation rate in the scaffold with increase in β-TCP content. Moreover, the presence of β-TCP served as physical crosslinking sites, which enhanced the stability of the network. All of the above results revealed that the degradation rate may be controlled by adjusting the β-TCP contents in the scaffold.

Results

Gelatin/sodium alginate composite hydrogels showed some elevations at certain places with folded structures. The incorporation of TCP in the hydrogel displayed agglomerated structures on the surface. TCP incorporated with gelatin/sodium alginate shows much less clotting time and higher percentage in hemostat.

Gelatin are the easiness of chemical modification and the commercial availability of samples with different physicochemical properties. A biodegradable hydrogel of gelatin for the controlled release of BMP to succeed in inducing bone regeneration. The gelatin/β-TCP composite scaffolds, which have compositional and structural features close to natural bone ECM, supported by adhesion, spreading, and proliferation and ALP activity.

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Fig 1

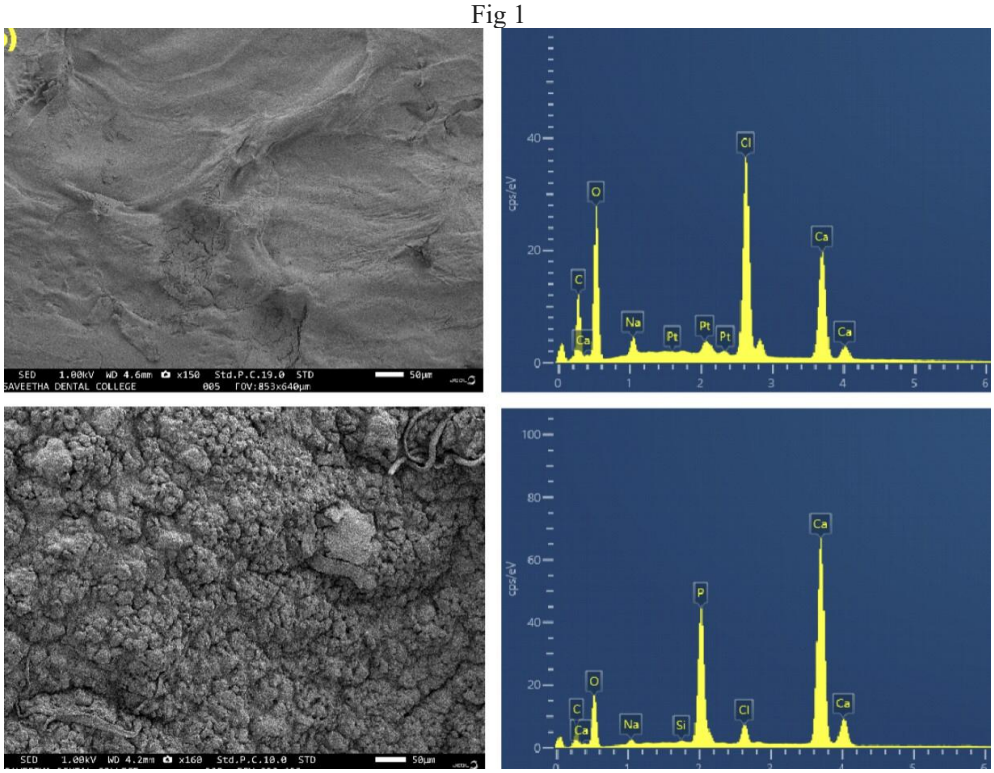

Fig 2

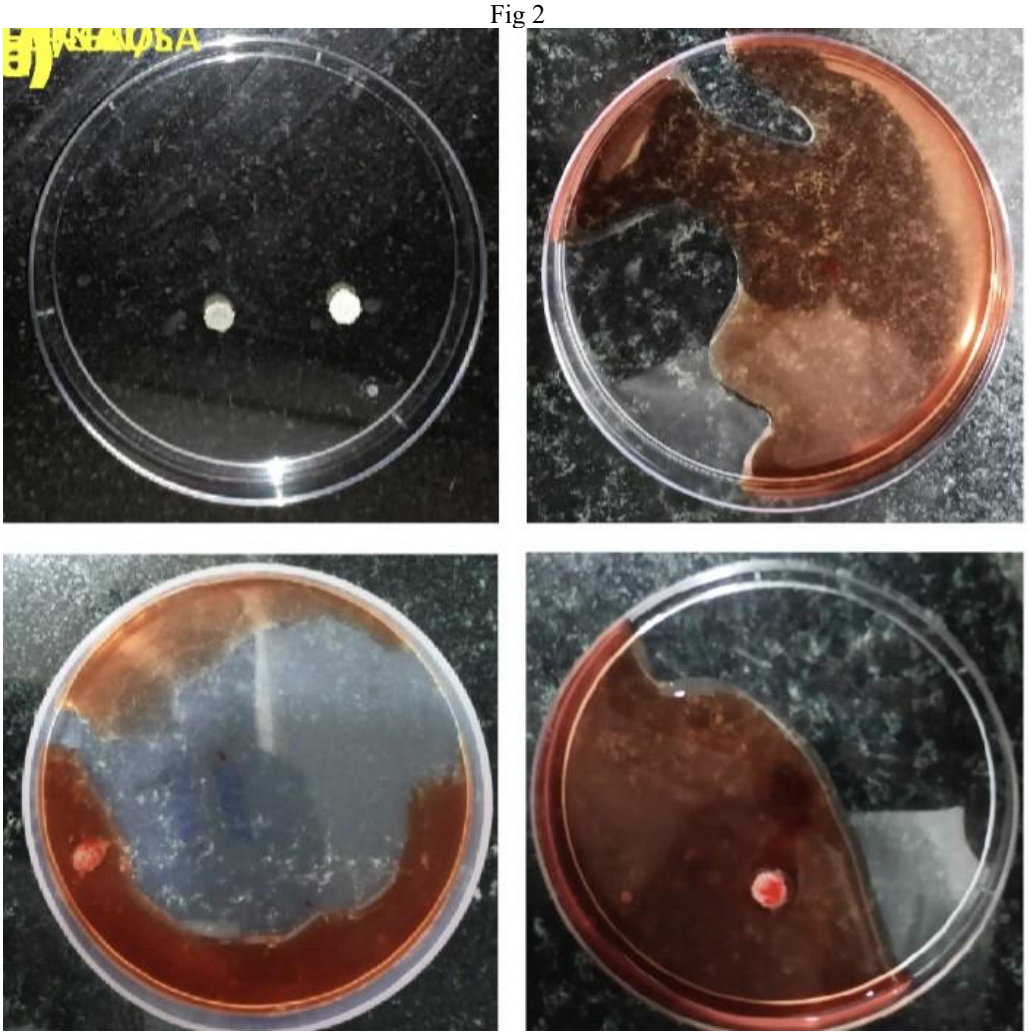

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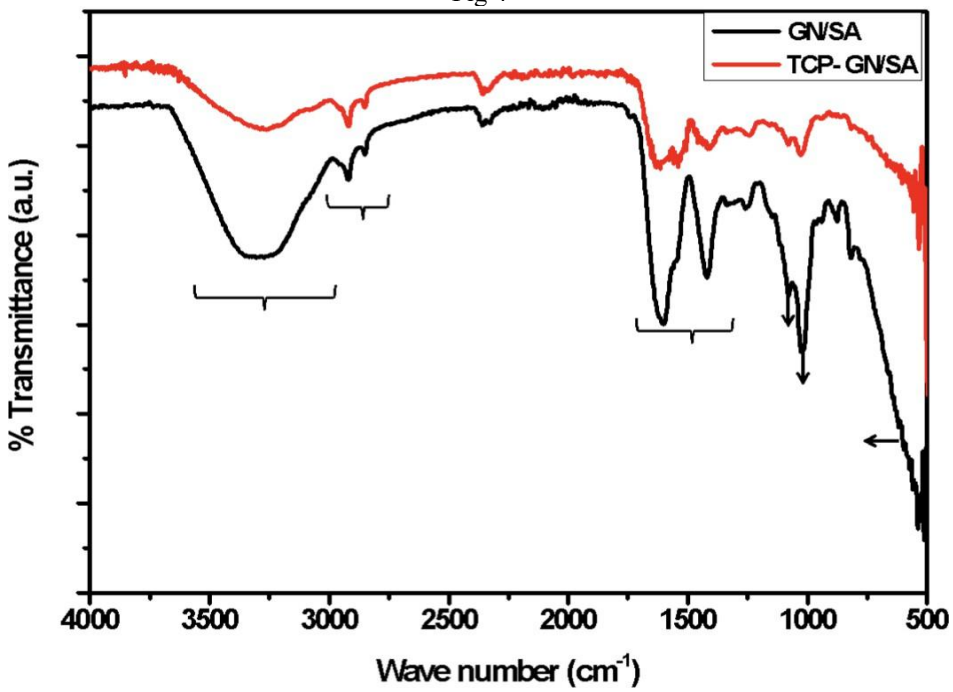
Fig 3

S.No.	Sample	Clotting time (MIN)	% Hemostat
1	Control	12	-
2	GN/SA	9.5	88.24
3	TCP-GN/SA	8.1	90.31

Fig 4



Discussion

Gelatin/β-TCP composite scaffolds induced osteogenic differentiation in vitro by activating Ca2+-sensing receptor signalling. The gelatin/β-TCP composite exhibited more extensive osteogenesis and has great potential in the practical application in orthopedics and dentistry, such as guided bone regeneration membranes in periodontal pockets.Chitosan as a biopolymer receives much attention due to its biocompatibility and biodegradability and its structural similarity with glycosaminoglycans. Moreover, chitosan can interact with growth factors, receptors, and adhesive proteins . Further, the unique features of chitosan have been appreciated in bone tissue engineering, prepared chitosan/calcium phosphate composites for studying the effect of chitosan on the crystal phase of CaP . investigated the two bone matrices (Col-CS-HA and Col-HA) and found that chitosan helped in osteoblast adhesion and proliferation *in vivo* by altering the surface chemistry, which in turn would accelerate the process of bone regeneration. Gelatin protein, derived from partial hydrolysis of collagen, has been extensively used in the orthopaedic field . Ease of handling of gelatin in water based environment and its stable interaction with β-TCP nanoparticles in water based suspension promoted its use over water insoluble collagen.

Further, possession of properties like its biological origin, biodegradability, gelation tendency in water media and commercial availability at low cost, it is considered to be potential candidate material for bone tissue engineering It contains free carboxyl groups on its backbone and has the potential to blend with chitosan to form a network through hydrogen bonding. The nano β-TCP composite porous scaffolds were successfully fabricated using freeze drying method. The prepared scaffolds were highly porous, with porosities larger than 80%, and had interconnected pores. Both FESEM image and porosimetry data showed that pore size of the nano-composite scaffold could be tailored by changing the β-TCP content in the scaffold. Increasing β-TCP content into the chitosan/gelatin (CG) matrix significantly improved mechanical properties and up to 30 wt% addition of β-TCP resulted in a higher compressive strength in scaffolds.

The GCT30 scaffolds showed the highest compressive strength of 2.45 MPa which eventually matches with the lower range mechanical properties of cancellous bone. Protein adsorption experiments showed GCT30 scaffold had higher

protein adsorption capacity with increasing β-TCP content up to one week of incubation. Furthermore, GCT30 scaffolds exhibited good biocompatibility and promoted better cell proliferation and MSC's differentiation into osteoblast than only neat gelatin-chitosan scaffold up to 14 days of cell culture. Also, GCT30 composite scaffold was found to promote new blood vessels formation and skin tissue regeneration *in-vivo*. also it shows that GCT30 scaffold can serve as a potential candidate for bone tissue regeneration using an artificial bone substitute material.

Conclusion

The scaffold having multi-sized pores were successfully fabricated using template coating and freeze drying methods. The gelatin-coated scaffold was fabricated uniformly by dip coating. The compressive strength of the β-TCP scaffold was enhanced about five times by gelatin coating. The scaffold having multi-sized pores resulted in improved cell differentiation, and gelatin coating enhanced the cell proliferation and differentiation. This study provides significant data regarding the mechanical and biological properties of the β-TCP scaffold according to the multi-sized pores and gelatin coating.

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