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Original Article

In vitro Characterisation of an Injectable Composite Graft Material Synthesised from Collagen Extracted from Fish Scales and Eggshell-Derived Hydroxyapatite

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ABSTRACT

Aim/Background: Collagen and hydroxyapatite have been used as scaffold for bone repair encouraging cell adhesion and growth. Composites synthesized from hydroxyapatite and collagen mimic biomechanical properties of natural bone. Extensive efforts have been made to fabricate graft materials from natural sources to mimic biochemical and biomechanical properties of natural bone. The present study is aimed to synthesise a composite graft from eggshell derived hydroxyapatite and fish collagen and characterize the novel material. **Materials and Methods:** The composite graft material was prepared using eggshell derived hydroxyapatite (60%) and fish derived collagen (40%) by lyophilisation. The prepared novel material was characterised using FTIR, XRD and SEM Cell viability was assessed using MTT assay. The material is then mixed with a suitable binder (glycerol). **Results:** The FTIR and XRD analysis confirms a composite material of hydroxyapatite- collagen composite graft. The ratio of hydroxyapatite to collagen was 60: 40. Crystallinity of the hydroxyapatite was not disrupted by the collagen. The collagen particles agglomerated with each other as well as on hydroxyapatite particles according to SEM. The composite graft was found to be noncytotoxic. **Conclusion:** In the present study eggshell derived hydroxyapatite and fish collagen has been used to synthesize a composite graft. Glycerine has been used as a binder to improve the properties. The prepared novel composite material exhibited good cell viability and may be effectively used as an alternative to conventional bone substitutes.

Keywords Bone graft, Composite, Eggshell, Hydroxyapatite, Fish Collagen, Characterisation.

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INTRODUCTION

Natural bone is a heterogeneous composite comprising principally of Hydroxyapatite (HA) and collagen. The excellent mechanical properties of bone result from its biphasic and ultrafine structural design of HA crystals embedded into the collagen matrix. With the ensuing increase in periodontal diseases and defects, there is an increasing demand for newer biomaterials to help regenerate soft and hard tissues and restore the function and aesthetics of teeth and surrounding tissues.[1] Currently, autogenous grafts are represented as gold standard to restore bone in the surgical management of osseous defects.[2] Collagen is considered an essential component of the extracellular matrix in bone tissue, which degrades easily, has strong plasticity and low immunogenicity, and has excellent biocompatibility, cell adhesion, and osteoconductivity. However, unmodified collagen alone may not produce acceptable investigational results and has poor physicochemical and mechanical properties. Hence, bone tissue engineering scaffolds which are biomimetic are often prepared in conjunction with bioactive materials with the combination of ceramic substances and collagen materials, which make ideal biomaterials for bone graft substitutes.[3,4]

HA is a commonly used bioactive ceramic material in bone tissue engineering, and it was found to be one of the safe, potential

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alternative materials due to its non-cytotoxic properties, biocompatibility, and comparable mineral phase with respect to the inorganic composition of bone. HA can be developed through both synthetic and natural sources. Synthetic sources often involve tedious procedures in the preparation, and changes in the crystalline phase in the HA during the synthetic procedure are also possible. Hence, the naturally derived HA from various animal bones, eggshells, and fish scales have gained much attention for the preparation of HA. Eggshells are one of the natural resources that can be used to synthesize HA. Meanwhile, HA simulates cancellous bone in its initial mechanical property; it becomes brittle under tension and shear but is resistant to compressive loads.[5]

Human bone exhibits distinctive characteristics of collagen as well as HA, precisely rigidity and toughness, making it the best-suited material to provide a framework for the human body that is light but strong. On a microscopic level, the needle-shaped inorganic crystals are essentially arranged along the length of the collagen fibrils, whose primary function is to connect and constrain the longitudinal fibers so that they are stable under compressive and bending loads.[6] Presently the commercially available synthetic bone grafts seem to lack distinct osteoinductive and mechanical properties or are relatively expensive involving extensive procedures during synthesis. Hydroxyapatite derived from eggshells and collagen derived from fish scales are natural sources which are easily available, cost effective and a biocompatible alternative to conventional bone grafts which have proved their potential for bone regeneration.[7,8]

There are numerous studies on the use of eggshell derived hydroxyapatite and fish collagen used separately for the purpose of bone regeneration but very few on the combination of these two materials. Addition of glycerine as a carrier provides an added benefit on their handling properties, shelf life and significant enhancement in bone healing.[9,10]

In the current study, we have developed HA-Collagen composite materials where HA is from eggshell sources and collagen is from the fish scales. Further, FT-IR, SEM, XRD, and biocompatibility studies characterized the developed composite materials.

AIM

Aim of the study is to synthesize and characterise a composite graft from eggshell derived hydroxyapatite and fish collagen.

MATERIALS AND METHODS

Synthesis of Collagen from Fish Scale

Collagen was extracted from scales of Rohu fish (*Labeo rohita*) using a laboratory standardized protocol. Briefly, scales were subjected to a series of washing protocols, initially with 10% NaCl in the ratio of 1:10 (scale: media) for 24 hr to remove the adhering debris and slime. Demineralization was carried out with 0.4 M HCl in the ratio of 1:10 (scale: media) for 3 hr. Further, it was treated with 0.5 M acetic acid at pH 2.5 in the ratio of 1:8 (scale: media) for 48 hr. Insoluble portions were removed by filtration, and the supernatant was collected. The residue was extracted with 1% Pepsin in 0.5 M acetic acid for 48 hr in a ratio of 1:8 (scale: media). Insoluble portions were removed by filtration, and the supernatant was collected. Both the acid-soluble and pepsin-soluble fractions were pooled. Soluble collagen was collected by salting out of the pooled filtrate using NaCl to a final concentration of 0.9 M and kept undisturbed for 10 hr. Collagen was collected as a pellet by centrifugation at 8000 rpm for 1 hr. Further, the pellet was re-dissolved in 0.5 M acetic acid. This salting out-salting in process was repeated two more times. The salt was removed from the final suspension by dialysis (molecular weight cut off range 14 kDa, Dialysis tubing cellulose membrane, Sigma Co, USA, initially against 0.5M acetic acid for 48 hr under stirring at 150 rpm, and subsequently with distilled water until the suspension reached a neutral pH. Purified collagen was collected by centrifugation at 4°C for 1 hr and freeze-dried. The entire extraction processes were carried out at 4°C.

Synthesis of eggshell-derived HA

Domestic chicken eggshells were washed thoroughly, and the inner membranes separated. The shells were then dried, powdered in a domestic blender, and passed through a sieve (BSS 85-0.18 mm). 1 gm of powdered eggshell was heated at 900°C in a muffle furnace for 2 hr.

$$Ca\,CO_3\ (Eggshell) \rightarrow \Delta \rightarrow CaO + CO_2$$

Due to its strong hygroscopic properties, calcium oxide was instantly converted to calcium hydroxide after exposure to the atmosphere.

$$CaO + H_2O \rightarrow \Delta \rightarrow Ca\,(OH)_2$$

A 0.3 M suspension is prepared by weighing calcium hydroxide, mixing it with distilled water, and reacting with diammonium hydrogen phosphate solution (0.5M) at a stochiometric ratio of Ca/P= 1.67. The impurities were separated by rinsing multiple times with distilled water and then dried in a hot oven at 100°C to produce hydroxyapatite crystals of which average particle size was found to be 56.50 µm.[11]

Synthesis of HA-Collagen biocomposites

Biocomposites were developed by mixing 60% HA and 40% collagen substances to mimic the natural bone composite.[12] Lyophilisiation was used to combine the materials. A Bench top lyophileser was used (Labconco, USA) at temperature 80°C, vaccum level 0.02mbar, sample volume 5 mL and flask size of 15 mL. The procedure was carried out for 24 hr.

The product is mixed with a binder (glycerol). 6 g of eggshell-derived HA is mixed with 4 g of fish collagen powder.

The composite was homogenized using mortar and pestle for 60 min, followed by lyophilization. The ratio of HA to collagen is 60% to 40% by weight. The 10 mg of the composite material was mixed with glycerol before application to provide an ideal consistency. 6 mL of glycerine is added to provide an ideal consistency.

Chemical Characterization of HA-Collagen Composites

The resultant mixture was characterized using FTIR, XRD and SEM. Characterization of the different functional components of the material was analysed by FT-IR (Shimadzu) spectra analysis in the range of 4000 to 500 cm^{-1}. Approximately 45 scans were conducted at a resolution of 2-4 cm^{-1}. The HA-Collagen biomaterial was analyzed using Rigaku Miniflex 600 (5th generation) with up to 40 kV X-ray generation, and a current of 15 mA.

Biocompatibility assessment

L929 murine fibroblast cells were used for the study which were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% glutamine and 1% antibiotic-antimycotic solution. Cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere throughout the experiments. Cell viability of the HA-collagen composite material was assessed using Methyl Thiazolyl Tetrazolium (MTT) assay. Cells were seeded onto 96-well microtiter plates at a seeding density of 5000 cells/well. It was allowed to attach overnight at 37°C and 5% CO_2 in a humidified condition. After adherence, varying concentrations (0, 12.5, 25, 50 and 100 µg/mL) of the sample were added and incubated for 24 hr. After 24 hr of incubation, the media was decanted, after which MTT reagent (1 mg/mL) was added and incubated at 37°C for 4 hr. MTT solution was solubilized using dimethyl sulfoxide. The absorbance was recorded at 570nm using microplate reader (Fluo STAR Omega, BMG Labtech). The viable cells were calculated in percentage with respect to untreated control. The experiment was repeated thrice.[13]

RESULTS

Biocomposites were developed by mixing 60% HA and 40% collagen substances to simulate the natural bone composite. Figure 1 shows the preparative procedure of HA-collagen composites. Glycerol was included in the HA-collagen composites to maintain the slurry and injectable form.

Chemical Interaction Study of HA-Collagen by FT-IR

Fourier Transform Infrared Spectroscopy

The bands at 3263 cm^{-1} correspond to OH and the peaks at 1034 cm^{-1} represents the phosphate group indicating the presence of hydroxyapatite. The N-H groups correspond to 2939 cm^{-1} and 2886 cm^{-1}. CH stretching is observed at 1649 cm^{-1} and 1409 cm^{-1} stretching corresponds to the carboxyl group which represents collagen (Figure 2). There were no intensive bands in the spectra range of 1800-2800 cm^{-1}, a characteristic of all biological materials. Peak at 1649 cm^{-1} represents the amide I bend which reconfirms the presence of collagen. Two near vibrations bands at

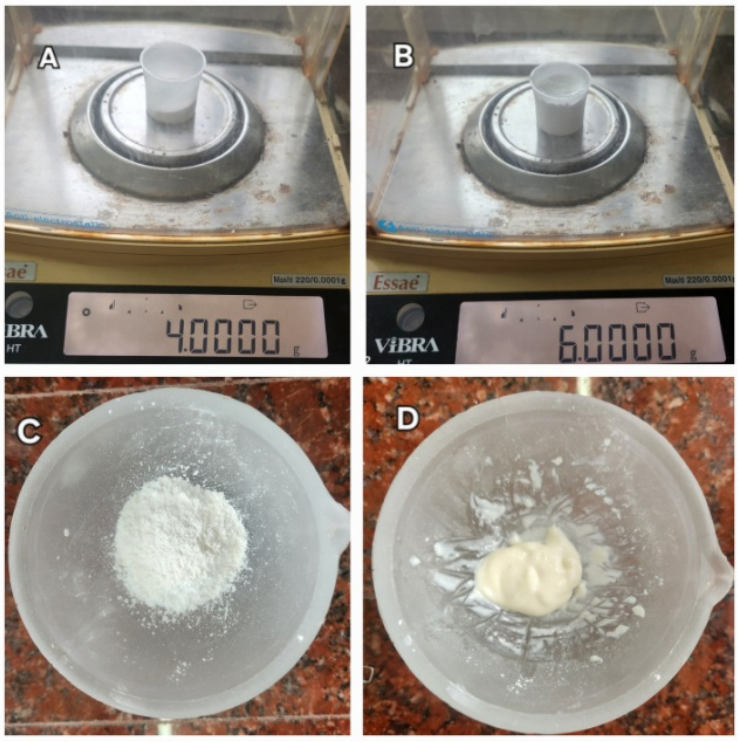

Figure 1: A) Fish collagen powder(4g), B) HA(6g) C) Lyophilized mixture, and D) Composite material mixed with 6 mL glycerol.

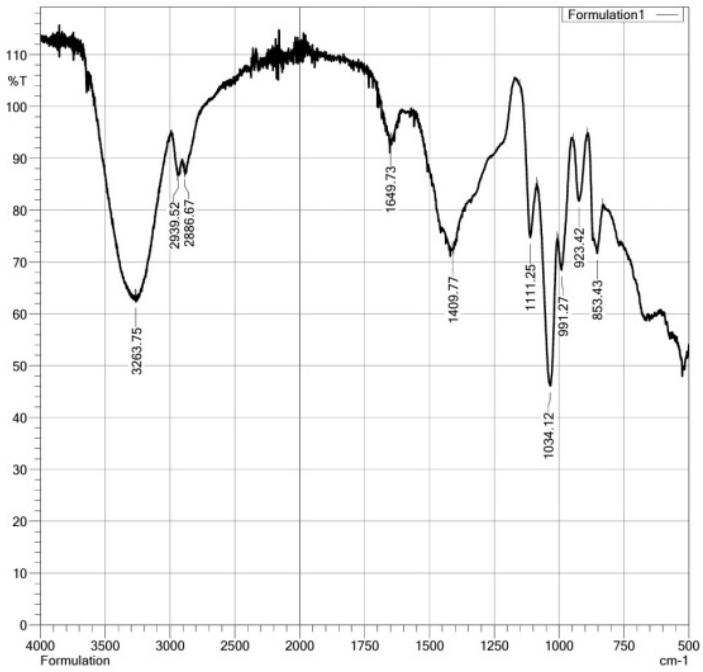

Figure 2: FT-IR spectra of HA-Collagen composite.

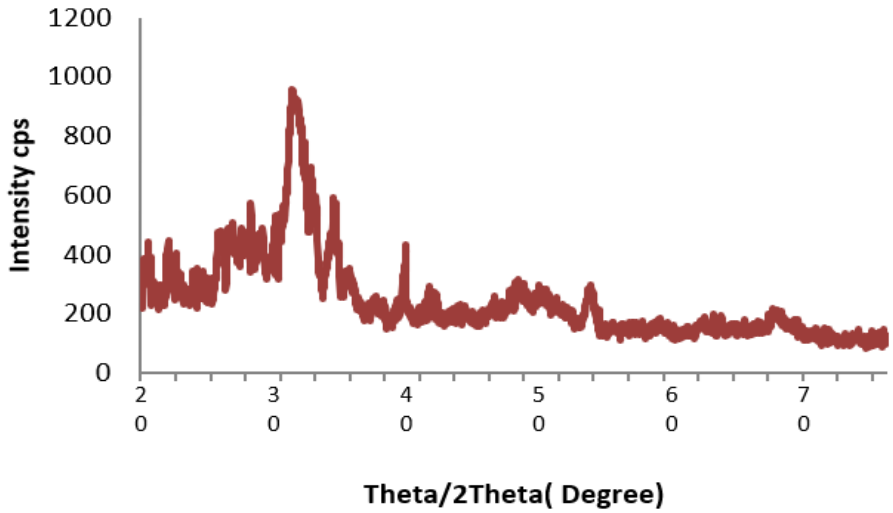

Figure 3: X-ray diffraction spectrum of HA-Collagen composite.

2900 cm^{-1} arise from collagen, most probably from the vibration of the OH group.

X-ray Diffraction (XRD)

Strongest peak was recorded at 31.4 (Figure 3). The X-ray diffraction pattern of HA and collagen mixture showed strong characteristic patterns of HA. Pure collagen is amorphous and shows no characteristic peaks. Collagen did not alter the crystallinity of HA. The results of XRD patterns include the organic and ionic species. The synthesized compound matched JCPDS 09-0432.

Scanning Electron Microscopy

The SEM images suggest that the particles of HA were combined with collagen, and collagen particles agglomerated and stuck with each other and HA particles, which might be a reason for increasing the particle size and making it difficult to calculate the exact particle size. Infact the collagen particles also deposited on the crystalline hydroxyapatite (Figure 4C, D). The hydroxyapatite particles were larger in size but less than 100 µm. The average size of the collagen particles as estimated by dynamic light scattering (Horiba ScientificSZ-100) was 327 nm but due to their agglomeration it seemed to appear larger in SEM (> 1µm) (Figure 4 E).

Biocompatibility studies

At varying concentrations in µg/µL of the material (12.5,25,50,100), a cell viability of 79.76, 113.49, 121.01 and 106.8273 was recorded (Table 1). The material favoured cell growth and multiplication (Figure 5).

DISCUSSION

The novel composite graft synthesised here was derived from two distinct natural raw materials, namely collagen synthesised from fish scales and hydroxyapatite synthesised from chicken eggshells. The characterisation of the biocomposite was done using FTIR to determine the functional groups and revealed hydroxyl (OH-), phosphate (PO_4) of hydroxyapatite and carboxyl and amide (NH) groups in collagen. The novel material was

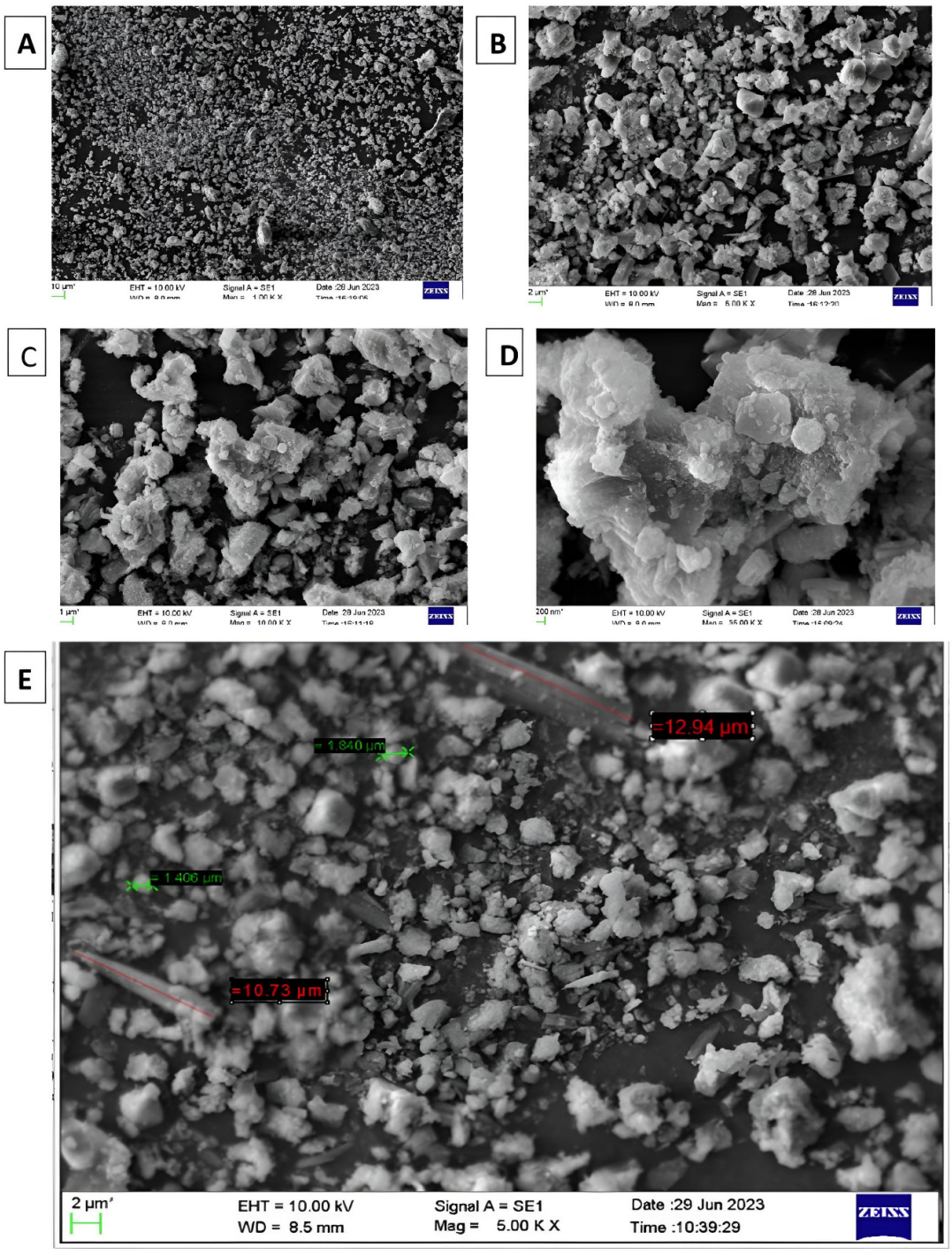

Figure 4: SEM images taken at different magnifications A) 1K, B) 5K, C) 10 K and D)35 K E) Needle shaped Hydroxyapate crystal of particle size 10.685 µm,12.261 µm, Collagen particles of size 1.84 µm and 1.406 µm.

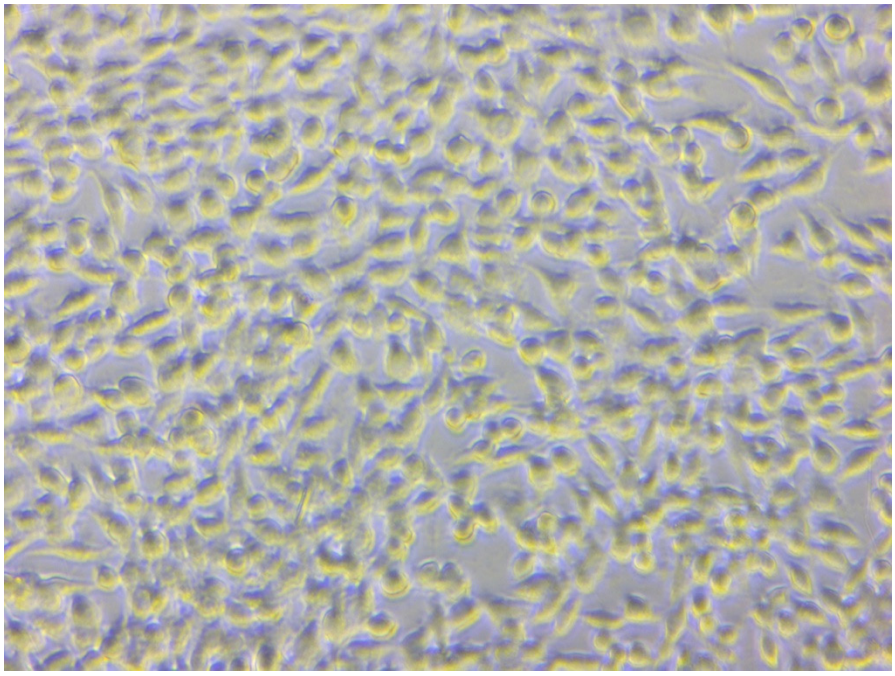

Figure 5: (A) Cell viability of HA-collagen composites and (B) microscopic images of the cells treated with HA-collagen composites.

Table 1: Cell Viability Values (MTT assay).

Concentration in µg/µL	Cell Viability (%)	Standard deviation
12.5	79.76	1.938
25	113.497	3.487
50	121.01	2.998
100	106.82	2.597

found to be biocompatible. The collagen particles had an effect on the particle size and on needle shaped hydroxyapatite crystals due to agglomerating nature. Incorporation of glycerine improved its consistency and facilitated its use in injectable form and did not affect the biocompatibility.

Collagen can form molecular scaffolds that can provide a framework for tissues and organs. Simultaneously, it can bind to receptors at cell surface and modulate their activities.[14] Its regenerative role is by regulating the dynamic balance, ultimately synchronizing the development of tissue or organs. Collagen also exhibits excellent biocompatibility, biodegradability, absorbability, and porosity, facilitating directed cell growth and a metabolic interconnection structure, making it one of the most versatile biomaterials. Its compromised physical properties and rapid degradation rate restricts its use as discrete biomaterial. Currently, composite materials containing collagen that stimulate osseous and cartilaginous tissues are under development. HA and calcium phosphate are bone inducers that often add active ingredients to collagen.[15,16]

Wahl DA, *et al.* suggested that HA-collagen composites resulted in early bone regeneration & increased density of the formed bone.[17] In the current study, different sources of HA and collagen were used. Araujo M, *et al*, in a study on healing of extraction sockets in dogs also found fresh bone formation 3 months post surgically, specially in the cortical regions after using HA-collagen composite (Bio-Oss Collagen).[18] Liu, *et al.* used a solid-liquid phase separation method to synthesize a highly porous collagen-HA scaffold. The synthesized scaffolds were a 3D interconnected fiber microstructure with 50-150 µm pore sizes, and HA particles were scattered uniformly among collagen fibers. The composite displayed superior mechanical properties compared to pure collagen. The composite had superior biocompatibility, and the addition of HA did not affect its histocompatibility.[19] Shui Jin, *et al.* fabricated fibrous membranes for guided bone regeneration using fish collagen and HA-reinforced poly (lactide-co-glycolide). The developed composites significantly increased bone tissue regeneration.[20]

Elline, *et al.* characterized a hydrogel composite scaffold using eggshell nano HA, bovine collagen, and epigallocatechin-3-gallate. The FT-IR results exhibited intermolecular bonds in the novel scaffold. According to the XRD analysis, the crystal structure and size of HA were affected by collagen. SEM analysis revealed the presence of a microporous scaffold with well-distributed HA particles in the collagen pores. The scaffold was found to be non-toxic with viability of the cells above 50%.[21]

Antoniaac, *et al.*, in their work, developed composite scaffolds of collagen (10%)-HA (90%) and collagen(10%)-HA(80%)-Mg(10%). The composites were prepared using lyophilization. FTIR spectra revealed characteristic bands at 630 cm^{-1}, 960 cm^{-1}, 1090 cm^{-1}, and 1024 cm^{-1}, representing vibrations of hydroxyl groups and phosphate groups of HA. Bands for

amide I (1649 cm^{-1}), amide II (between 1500 and 1590 cm^{-1}), and pyrrolidine rings (1450 cm^{-1}) were also registered, which depicted collagen. The results are coherent with currently prepared HA-collagen composites. SEM analysis revealed a homogeneous distribution of HA particles adherent to the collagen matrix. An interconnected pore structure was also identified.[22]

When a modified electrospinning procedure was used to formulate homogeneous HA-collagen composite sols in an organic solvent, composite fibers showed a uniform and continuous morphology, with a well-organised sequencing of the needle-like HA nanocrystals along the collagen fibers without any agglomeration.[23] In our study, the collagen particles were impregnated and stuck with each other, as well as on HA particles, thus increasing uniformity. Son, *et al.* increased the collagen content and increased the average particle size of the mixed powder.[24] Chuysinuan, *et al.* fabricated a scaffold for bone regeneration using HA-incorporated fibroin-alginate composite injectable hydrogel. Eggshell biowaste was used to synthesize HA. Fibroin was extracted from Bombyx mori cocoon. Decreased pore size was noted in the novel scaffold compared with pure alginate hydrogel. The primary cytotoxicity test indicated that the material was non-toxic.[25]

Samsell, *et al* used glycerol for preservation of freeze-dried bone allografts and found that it had similar osteoconductivity and biocompatibility to frozen and freeze-dried samples.[10] Glycerol also enhanced the bone forming ability.[9] Our study also proves that addition of glycerol did not have a negative impact on the biocompatibility of the material and improved the handling. Brittleness, fragility and difficulty in molding being one of the major shortcomings of hydroxyapatite containing bone substitutes, the improvement in manuvourability will positively impact their ability to regenerate bone.

Mechanical testing of this material including thermal behaviour, compressive strength, resistance to mechanical forces needs to be analysed which is lacking in this study. Further *in vivo* studies will be required to confirm its osteoinductive properties as well as the *in vivo* biocompatibility of the material.

CONCLUSION

HA is a biocompatible substance that has been extensively studied in bone tissue engineering applications. In the present work, homogenously blending and freeze lyophilization methods prepared an HA-collagen bio-composite graft. The prepared material is mixed with glycerol to further improve the manoeuvrability and shelf life. Close interaction between HA and collagen was observed at the interface of the phases. Proper utilization of eggshell-derived HA and fish scale-derived collagen bio-composite for HA-collagen can potentially be used as a bone graft substitute in dentistry and orthopaedic applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HA: Hydroxyapatite; **FTIR:** Fourier Transform Infrared Spectroscopy; **XRD:** X-ray Diffraction; **SEM:** Scanning Electron Microscopy.

ETHICAL STATEMENT

This research dheres to ethical research standards. All sources are properly cited to acknowledge the original authors' contributions. No data fabrication, falsification, or plagiarism has been involved in the preparation of this manuscript. The authors have ensured that the work is original and does not infringe on any existing copyrights or intellectual property rights.

SUMMARY

In this original research, we have synthesized and characterized a novel graft material. The material is a composite which contains hydroxyapatite derived from domestic chicken eggshells and collagen derived from fish scales. Hence two natural resources have been used to prepare this novel graft material making it more economical and easily available. The material has been characterized by FTIR, XRD and SEM. *In vitro* biocompatibility studies also have been done. We are continuing further studies on animal models to see the ability of the material to form new bones.

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