ISSN 3060-4567

# BIOACTIVE QUERCETIN-CROSSLINKED COLLAGEN/HYDROXYAPATITE SCAFFOLDS FOR BONE TISSUE ENGINEERING

***M. A. Kodirov***

*Assistant, Department of Biological Chemistry*

*Andijan State Medical Institute*

***Abstract:*** *Tissue engineering is essential in dental implantology. Collagen is the main structural protein of the human extracellular matrix (ECM), is widely used in various areas of tissue engineering. We prepared collagen (Col)/hydroxyapatite (Hap)/ quercetin (QU) constructs with enhanced bone-forming capability. The Col/HAp composite scaffolds were prepared by subjecting collagen sponges to five successive cycles of alternate immersion in calcium and phosphate ion solutions, with exposure times of 20 and 60 min, respectively. After mineral deposition, the sponges were heated at 56 °C for 48 h.*

***Keywords:*** *collagen/hydroxyapatite composite; quercetin; alternate immersion method; hydroxyapatite precipitation; bone substitute materials; dental implantology.*

**Introduction.** In nature, collagen provides mechanical support and protection of tissues from damage. It consists of three polypeptide chains (glycine, proline , and hydroxyproline ) that form a left-handed α-helix, which are then combined to form a right-handed triple helix — the characteristic structure of collagen [1].

Currently, 28 types of collagen are known, of which types I and II are most widely used in biomaterials.

Collagen is a glycoprotein, a fibrillar protein that forms the basis of connective tissue in the body (tendon, bone, cartilage, dermis, fascia, etc.) and provides its strength and elasticity. Collagen is found in animals, Absent in plants, bacteria, viruses, protozoa, and fungi, collagen is the main component of connective

ISSN 3060-4567

tissue and the most abundant protein in mammals, comprising 25% to 45% of the total body protein. Collagen synthesis is highly energy-intensive and occurs only in animals that utilize oxygen. The emergence of collagen enabled the creation of both external and internal skeletons and the dramatic increase in animal size during the Cambrian Explosion. The product of collagen denaturation is gelatin. The denaturation temperature of collagen macromolecules is close to the fibrillogenesis temperature. This property of the collagen molecule makes it extremely sensitive to mutational substitutions.

Fibrillogenesis is the formation of collagen fibers in connective tissue by the assembly or fusion of fibrils - thin protein thread-like structures within cells and tissues of the human body into bundles. Fibrillogenesis is essential for implant engraftment and the creation of a strong, properly functioning masticatory system. The stronger the collagen fibers formed during fibrillogenesis, the stronger the connective tissue. A collagen molecule is a right-handed helix of three α-chains. This formation is known as tropocollagen. One turn of the α-chain helix contains three amino acid residues. The molecular weight of collagen is approximately 300 kDa , its length is 300 nm , and its thickness is 1.5 nm. The primary structure of the protein is characterized by a high content of glycine , a low content of sulfur-containing amino acids and the absence of tryptophan . Collagen is one of the few proteins of animal origin that contain residues of non-standard amino acids : about 21% of the total number of residues are 3-hydroxyproline, 4-hydroxyproline and 5-hydroxylysine . Each of the α-chains consists of triads of amino acids. In triads, the third amino acid is always glycine, the second is proline or lysine , and the first is any other amino acid except the three listed.

Collagen exists in several forms. The basic structure of all types of collagen is similar. Collagen fibers are formed by the aggregation of microfibrils, have a pink color when stained with hematoxylin and eosin and blue or green with various trichrome stains, and stain brown-yellow when impregnated with silver.

**Introduction.** *Cross-linking* of Col using physical or chemical methods is frequently performed to improve its durability and strength. The cross-linking

method is outlined in Supplementary Material A: Cross-Linking Method of Collagen (Col) (Supplementary Materials) The techniques related to this study were physical de-hydrothermal treatment (DHT) and chemical cross-link quercetin (QU). Col has also been combined with osteo-conductive hydroxyapatite (Hap) to produce composites used for bone regeneration in dental implantology and oral surgery [18–21].

**Experimental part.** *Preparation* of Col/Hap Composite Granules Col pellets (1 g) were dissolved in 28 mL of distilled water in a 50 mL polystyrene conical tube at 4 ºC. The acidic solution was neutralized using 0.1 N NaOH solution (6.5 mL) in three rectangular plastic plates (84 º 54 º 12 mm3) to achieve a Col gel pH of 7.5. The Col gel was frozen at 80 ºC for 12 h and freeze dried for 12 h. The resultant sponge was cross-linked using QU treatment at 25 ºC for 1 h.

The Col sponge was processed using an alternate immersion method (Figure 1). In brief, three sponge sheets produced from 1 g of pellets were cut into 0.5–1 mm granules using scissors. The granules were packed in nine Nylon meshes (Mesh Pack C, 60 mm º 80 mm; Sansho Co., Tokyo, Japan). The granules in the mesh were immersed in 100 mL of Tris-HCl buffered solution containing 200 mM CaCl2 (pH = 7.4) for 20 min at 37 ºC, blot dried using a paper cloth, immersed in 100 mL of Tris-HCl buffered solution containing 120 mM NaH2PO4 (pH = 9.3) for 20 min at 37 ºC, and blot dried using a paper cloth to complete one cycle of the alternate immersion method. The immersion cycle was repeated five times (AI 20 min 5Cy Col/Hap). The pH was adjusted with NaOH and HCl solutions using a pH/ion meter (F-24; Horiba Ltd., Kyoto, Japan). The composite sponges were produced by altering the immersion time of five cycle repetitions to 60 min (AI 60 min 5Cy Col/Hap). Two types of composite sponges were dried in a vacuum dry oven (VO-300; AS One) at 56 ºC for 48 h. Control collagen containing only granules without the use of alternate immersion was also prepared (Col control).

**Characterization of Biomaterials**

SEM/Energy-Dispersive Spectroscopy (EDS) and Scanning Electron Microscopy (SEM) Analyses

ISSN 3060-4567

The morphological and chemical properties of the outer surfaces of the two Col/Hap composite granule samples (AI 20 min 5Cy Col/Hap and AI 60 min 5Cy Col/Hap) were examined using SEM (SU8010; Hitachi High-Tech Co., Tokyo, Japan) and EDS (JSM-7100F; Joel Co., Tokyo, Japan) (n = 1) at an accelerating voltage of 10 kV. The outer and cross-sectional surfaces of three types of dried gelatin-infiltrated granules (Col control + AG, AI 20 min 5Cy Col/Hap + AG and AI 60 min 5Cy Col/Hap + AG) were examined (n = 1 for both) using SEM at an accelerating voltage of 15 kV after plasma coating with $OsO_4$.

**X-ray Diffraction (XRD) Analysis**

The crystallographic states of Col and Col/Hap composite granules (Col control, AI 20 min 5Cy Col/Hap, and AI 60 min 5Cy Col/Hap) were examined (n = 1 for all) using XRD (D8 Discover; Bruker AXS, Billerica, MA, USA), CuK_ radiation, and an accelerating voltage of 40 kV. Pure Hap produced by high-temperature sintering (187-37; Taihei Chemical Industry Co., Osaka, Japan) was used as standard for comparison.

**Fourier-Transform Infrared Spectroscopy (FTIR)**

The organic functional groups in Col and Col/Hap composite granules (Col control,

AI 20 min 5Cy Col/Hap, and AI 60 min 5Cy Col/Hap) were examined (n = 1 for all) using FTIR equipped with attenuated total reflectance attachment (Nicolet6700; Thermo Fisher Scientific, Waltham, MA, USA). Hap standard was also used for comparison.

**Results.** We prepared Col/Hap composite granules using the alternate immersion method and infiltrated them with cross-linked AG, followed by freeze drying. The growth factor b-FGF was impregnated into the prepared granules, which produced Col/Hap/AG/b-FGF constructs. The wet quaternary granules were implanted into rat cranial bone defects and evaluated using soft X-ray measurements and histological analysis. We self-prepared collagen membranes using chemical cross-linking to cover bone defects with and without filled constructs.

Despite its limitations, we can draw several important conclusions from our study of Col/Hap/AG/b-FGF granules. First, the self-prepared Col membrane provided a protective barrier and covering material for the defects. Second, instrumental analyses (SEM, SEM/EDS, XRD, and FTIR) showed that alternate immersion promoted small low-crystalline Hap precipitation on the collagen matrix. Third, the placement of the Col/Hap/AG/b-FGF construct increased new bone formation in the cranial bone defect compared to the defect without placement. Fourth, the newly formed bones extended from the bone defect edge and produced small island-like bones. Finally, the prepared construct (Col/Hap/AG/b-FGF) produced a moderate amount of new bone. Further materialistic studies are required to develop methods to improve bone formation.

## REFERENCES

1. Collagen type I: A versatile biomaterial. In *Novel biomaterials for regenerative medicine* (pp. 389–414). Springer.
2. Chowdhury, S. R., Busra, M. F. M., Lokanathan, Y., Ng, M. H., Law, J. X., Cletus, U. C., & Idrus, R. B. H. (2018).
3. Cimetta, E., Flaibani, M., Mella, M., Serena, E., Boldrin, L., De Coppi, P., & Elvassore, N. (2007).
4. Enhancement of viability of muscle precursor cells on 3D scaffold in a perfusion bioreactor. International Journal of Artificial Organs, 30(5), 415–428. Cittadella Vigodarzere, G., & Mantero, S. (2014).
5. Salvatore, L.; Gallo, N.; Natali, M.L.; Terzi, A.; Sannino, A.; Madaghiele, M. Mimicking the hierarchical organization of natural collagen: Toward the development of ideal scaffolding material for tissue regeneration. Front. Bioeng. Biotechnol. 2021, 9, 644595.
6. Shoulders, M.D.; Raines, R.T. Collagen structure and stability. Annu. Rev. Biochem. 2009, 78, 929–958.
7. Parenteau-Bareil, R.; Gauvin, R.; Berthod, F. Collagen-based biomaterials for tissue engineering applications. Materials 2010, 3, 1863–1887.

8. Ghomi, E.R.; Nourbakhsh, N.; Kenari, M.A.; Zare, M.; Ramakrishna, S. Collagen-based biomaterials for biomedical applications. J. Biomed. Mater. Res. B Appl. Biomater. 2021, 109, 1986–1999.

9. Koide, T. Designed triple-helical peptides as tools for collagen biochemistry and matrix engineering. Philos. Trans. R. Soc. Lond. B Biol. Sci. 2007, 362, 1281–1291.

10. Zhang, X.; Li, Q.; Wang, Z.; Zhou,W.; Zhang, L.; Liu, Y.; Xu, Z.; Li, Z.; Zhu, C.; Zhang, X. Bone regeneration materials and their application over 20 years: A bibliometric study and systematic review. Front. Bioeng. Biotechnol. 2022, 10, 921092.

11. Hsu, E.L.; Stock, S.R. Growth factors, carrier materials, and bone repair. Handb. Exp. Pharmacol. 2020, 262, 121–156.

12. Fiorillo, L.; Cervino, G.; Galindo-Moreno, P.; Herford, A.S.; Spagnuolo, G.; Cicciù, M. Growth factors in oral tissue engineering:

13. New perspectives and current therapeutic options. Biomed. Res. Int. 2021, 2021, 8840598.

14. Miyanaga, T.; Ueda, Y.; Miyanaga, A.; Yagishita, M.; Hama, N. Angiogenesis after administration of basic fibroblast growth factor induces proliferation and differentiation of mesenchymal stem cells in elastic perichondrium in an in vivo model: Mini review of three sequential republication-abridged reports. Cell Mol. Biol. Lett. 2018, 23, 49.

15. Shen, M.;Wang, L.; Feng, L.; Gao, Y.; Li, S.;Wu, Y.; Xu, C.; Pei, G. bFGF-loaded mesoporous silica nanoparticles promote bone regeneration through the catenin signalling pathway. Int. J. Nanomed. 2022, 17, 93–2608.

16. Takayama, S.; Murakami, S. Efficacy of FGF-2 in periodontal regeneration in a case of severe intrabony defect and furcation involvement with 15-month follow-up. Clin. Adv. Periodontics 2021, 11, 74–79.

17. Takei, Y.; Minamizaki, T.; Yoshiko, Y. Functional diversity of fibroblast growth factors in bone formation. Int. J. Endocrinol. 2015, 2015, 729352.

ISSN 3060-4567

18. Novais, A.; Chatzopoulou, E.; Chaussain, C.; Gorin, C. The potential of FGF-2 in craniofacial bone tissue engineering: A review. Cells 2021, 10, 932.