Bioactive Gum Arabic/κ-Carrageenan-Incorporated Nano-Hydroxyapatite Nanocomposites and Their Relative Biological Functionalities in Bone Tissue Engineering

Sumbul Mirza, Reshma Jolly, Iram Zia, Mohd Saad Umar, Mohammad Owais, and Mohammad Shakir*

Cite This: ACS Omega 2020, 5, 11279−11290

ABSTRACT: The present frontiers of bone tissue engineering are being pushed by novel biomaterials that exhibit phenomenal biocompatibility and adequate mechanical strength. In this work, we fabricated a ternary system incorporating nano-hydroxyapatite (n-HA)/gum arabic (GA)/κ-carrageenan (κ-CG) with varying concentrations, i.e., 60/30/10 (CHG1), 60/20/20 (CHG2), and 60/10/30 (CHG3). A binary system with n-HA and GA was also prepared with a ratio of 60/40 (HG) and compared with the ternary system. A rapid mineralization of the apatite layer was observed for the ternary systems after incubation in simulated body fluid (SBF) for 15 days as corroborated by scanning electron microscopy (SEM). CHG2 exhibited the maximum apatite layer deposition. Further, the nanocomposites were physicochemically analyzed by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), and mechanical testing. Their results revealed a substantial interaction among the components, appropriate crystallinity, and significantly enhanced compressive strength and modulus for the ternary nanocomposites. The greatest mechanical strength was achieved by the scaffold containing equal amounts of GA and κ-CG. The cytotoxicity was evaluated by culturing osteoblast-like MG63 cells, which exhibited the highest cell viability for the CHG2 nanocomposite system. It was further supported by confocal microscopy, which revealed the maximum cell proliferation for the CHG2 scaffold. In addition, enhanced antibacterial activity, protein adsorption, biodegradability, and osteogenic differentiation were observed for the ternary nanocomposites. Osteogenic gene markers, such as osteocalcin (OCN), osteonectin (ON), and osteopontin (OPN), were present in higher quantities in the CHG2 and CHG3 nanocomposites as confirmed by western blotting. These results substantiated the pertinence of n-HA-, GA-, and κ-CG-incorporated ternary systems to bone implant materials.

1. INTRODUCTION

A large number of critical bone injuries occur due to ageing, orthopedic diseases, hormonal imbalance, diabetes, and surgical resection, resulting in a remarkable loss of bone tissue impeding the natural healing capacity of bone. The interdisciplinary combination of engineering and life science has created a new domain of alternative biological substitutes for damaged tissues and organs, designed to restore their lost functions. However, the ideal regeneration and absolute restoration of the defective site still remain an unfathomable quest. Diverse approaches have been persistently optimized and reiterated in an attempt to fulfill the unmet clinical requirements. Then, the notion of artificial bone grafting came into play. The traditional methods of autografts and allografts have been rejected pertaining to their limitations in terms of scarring, grievous pain, donor site morbidity, and immunogenic rejection. Bone tissue engineering plays a focal role in subjugating these drawbacks by designing tissue constructs from a plethora of biomaterials with the intent of fulfilling the basic requirements of bone regeneration. It focuses primarily on some of the crucial parameters, such as mimicking the extracellular matrix of natural bone, adequate vascularization, biodegradability, osteoconductivity, and excrement removal. The process of bone development and remodeling are regulated by the association between the extracellular matrix, biological factors, cells, and biomechanical forces. Although there is a significant advancement in technological strategies for designing implantable materials, a remarkable increase in bacterial infection cases has been noted. This leads to a delay in the healing response, thus affecting the patient’s life critically. An arduous challenge is to overcome these mishappenings by selecting materials that are hemocompatible as well as antibacterial in nature. Many bone tissue
engineering strategies have been explored so far, but only some have been accepted for clinical use. These are mainly single-component strategies including cells, factors, or synthetic defect-filling materials, which are expensive and have side effects. Scaffolds utilizing growth factors have also been developed recently for successful bone repair and regeneration. The growth factor approach has been hampered by several complications, including high dose requirements, lower half-life, protein instability, higher costs, and undesired side effects. With these limitations of current material systems and the quest to overcome the existing shortcomings, we aimed to synthesize a biocompatible nanocomposite system with naturally derived components, which are both bioactive and economically favorable, in a view to expedite the process of bone tissue regeneration.

In this regard, a gamut of polymers (natural and synthetic) have attracted unprecedented attention in tissue engineering as they are processed easily and their properties can be tailored effectively. However, polymers cannot solely accomplish the requirements of an ideal scaffold, necessitating the incorporation of inorganic reinforcements.

Carrageenan (CG) is an anionic unbranched sulfated heteropolysaccharide consisting of repeating units of 3-linked β-D-galactopyranose and 4-linked α-D-galactopyranose. The three major categories of carrageenan include kappa (κ), iota (ι), and lambda (λ) carrageenan based on one, two, and three sulfate groups for each disaccharide unit, respectively. CG possesses numerous pharmaceutical properties, such as immunomodulation, anticoagulant, and antihyperlipidemic activities. It is also used in the food industry as a thickening and emulsifying agent. Numerous research studies have demonstrated that carrageenan is safe orally at maximum doses to neonates and adults, with no adverse effects on the immune system. Moreover, the lower the sulfate content in carrageenan the higher will be its gel strength, and the lowest ester sulfate is found in κ-carrageenan, making it most suitable to be used as a bone scaffold. Additionally, the structure of κ-carrageenan (κ-CG) is analogous to glycosaminoglycans, which are naturally present in the bone tissue and cartilage of humans. Some studies have also suggested carrageenan to have antitumoral activity, and thus, they have been documented for their anti-angiogenic effects in mice (Dias et al.). Another study reported apoptosis induction in human intestine (Caco-2) and hepatic (HepG2) cell lines, while no toxicity was observed in a corresponding normal cell (Ariff et al.). Their remarkable hydrophilicity and substantial bonding with enzymes and proteins along with their capability to aid cell proliferation and adhesion benefit κ-CG for scaffolding. On the other hand, acacia gum, also known as gum arabic (GA), is a branched polysaccharide comprising a galactose backbone with branches of glucuronic acid, arabinose, and rhamnose linked together. Gum arabic is easily dissolved in water to produce colloidal solutions, which are characterized by low stickiness and viscosity. According to the United States Food and Drug Administration (USFDA), gum arabic has the “Generally Recognized as Safe (GRAS)” status. These properties allow GA to be used in various routine applications. It also consists of salts, mainly K, Mg, and Ca. GA is used extensively in the food industry for microencapsulation, in pharmaceutics for complex coacervation processes, and is also used widely as a carrier for drug in different pharmacological and physiological experiments. The significant percentage of Ca present in GA aids in protecting against osteoporosis and contributes to its treatment. Moreover, its high Ca content also aids in increasing tooth remineralization. For scaffolding, the polymeric organic matrix must have inorganic reinforcements incorporated into it to mimic the nanocomposite framework of bone and to improve its biological and mechanical properties.

Hydroxyapatite, the basic inorganic component of bone, is often combined with polymers owing to its osteoconductivity, osteoinductivity, and its capability to directly adhere with hard tissues. Numerous research studies have been conducted to analyze the combination of nano-hydroxyapatite (n-HA) with several polymeric phases and the results suggested phenomenal biological and physicochemical properties for the composites. For instance, Kashiwazaki et al. synthesized biocompatible and biodegradable novel chitosan/hydroxyapatite nanocomposites with a porous structure by a co-precipitation and porogen-leaching method. Furthermore, Zhao et al. fabricated two kinds of biomimetic composites, chitosan–gelatin (CG) and hydroxyapatite/chitosan–gelatin (HCG), which displayed enhanced protein and calcium ion adsorption properties of HA in the CG polymer network, enhancing initial cell adhesion and long-term growth. Hadavi et al. have reported nanocomposites containing gum acacia and nano-hydroxyapatite and the results demonstrated favorable biocompatibility and a structure similar to that of the natural bone matrix. Similarly, Senthilarsan and Sakthivel also reported GA- and n-HA-containing nanocomposites, which were markedly promising in the formation of more distinguished orthopedic and dental implants. Apart from these composites, a large number of ternary nanocomposites have also been developed; e.g., Liuyun et al. synthesized composites containing nano-hydroxyapatite/chitosan/carboxymethylcellulose (n-HAp/CS/CMC) with different weight ratios by a co-solution method. These novel composites of n-HAp/CS/CMC exhibited good mechanical properties, adequate biodegradation rate, and bioactivity in simulated body fluid (SBF). Gum arabic was incorporated into chitosan–gelatin nanofibres by Tsai et al., developing a cost-effective and safer method having potential for bone tissue engineering. Feng et al. developed a collagen–hydroxyapatite/κ-carrageenan ternary nanocomposite in which a strong bonding interaction was shown between hydroxyapatite and carrageenan along with good biocompatibility and mechanical strength. Zamora-Sequeira et al. also fabricated conductive nanostructured materials based on poly-3,4-ethylenedioxythiophene and starch/κ-carrageenan for biomedical applications. Therefore, in line with the ongoing research to overcome the existing shortfalls, in the present work, ternary nanocomposites were constructed incorporating GA, κ-CG, and n-HA such that the concentration of n-HA was fixed and GA and κ-CG were varied in the n-HA/GA/κCG ratios of 60/30/10 (CHG1), 60/20/20 (CHG2), and 60/10/30 (CHG3). Besides, we have also synthesized a binary system consisting of n-HA and GA in the ratio of 60/40 for comparing and analyzing their properties with those of the ternary nanocomposite systems. All of these nanocomposite formulations were characterized with Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and X-ray diffraction (XRD) for evaluating their morphological characteristics. Furthermore, their in vitro biocompatibility and antibacterial activity were also evaluated with a MG63 cell line and the possibility of applying them as successful bone constructs was explored.
2. RESULTS AND DISCUSSION

2.1. SEM. The morphological characteristics of the as-synthesized nanocomposites were evaluated using SEM micrographs. As shown in Figure 1a1–d1, HG exhibited a smooth surface with compact edges, while the ternary nanocomposites CHG1, CHG2, and CHG3 revealed a relatively rough surface. Among the ternary systems, it was observed that CHG2 possessed the maximum surface roughness and better interconnectivity, facilitating cell attachment and proliferation.37 Moreover, the SEM results were further supplemented by atomic force microscopy (AFM) analysis, which revealed a rougher surface for the ternary systems as Figure 1.

Figure 1. SEM images of HG (a1), CHG1 (b1), CHG2 (c1), and CHG3 (d1) and their respective SBF study after 15 days (a2–d2) and 30 days (a3–d3).

Figure 2. Transmission electron micrographs of HG (a), CHG1 (b), CHG2 (c), and CHG3 (d) nanocomposites.
compared to the binary nanocomposite (Supporting Information, Figure S1). Therefore, it was confirmed that the addition of κ-CG into the binary system enhanced its bioactivity, and the nanocomposite, CHG2, was found to be a suitable bone scaffold.

2.2. TEM. The internal structure of the fabricated nanocomposites was further elucidated by TEM analyses. The nanocomposite, HG, consisted of particles coalesced into a cluster forming an aggregate (Figure 2). Upon addition of κ-carrageenan, it was observed that the ternary system exhibited a distributive nature of particles, which were rod shaped, and the most homogeneous dispersion was seen in the CHG2 nanocomposite, envisaging enhanced mechanical characteristics.

2.3. FTIR. The structural determination of the nanocomposites was carried out by FTIR spectroscopy. In the FTIR spectrum of HG, shown in Figure 3a, the band at 3400–3450 cm\(^{-1}\) was assigned to the OH stretching mode present in both gum arabic and hydroxyapatite. The peak at 1641 cm\(^{-1}\) was attributed to the COO\(^{-}\) symmetric stretching vibration of gum arabic. The characteristic peaks of hydroxyapatite were seen at 1036 cm\(^{-1}\) for the phosphate stretching vibration as well as at 603 and 566 cm\(^{-1}\), corresponding to the phosphate bending vibration. In the

Figure 3. (a) FTIR spectra of CHG1, CHG2, CHG3, and HG nanocomposites. (b) XRD patterns of CHG1, CHG2, CHG3, and HG nanocomposites.

Scheme 1. Possible Interaction between Nano-Hydroxyapatite, Gum Arabic, and κ-Carrageenan
ternary system, a prominent characteristic peak of the sulfate group was observed at 1392 cm$^{-1}$, confirming the presence of $\kappa$-carrageenan. Additionally, all other peaks that were observed in HG were present in all three ternary nano-composites. In CHG1 and CHG3, the intensity of the characteristic peaks at 1392, 603, and 566 cm$^{-1}$ was higher as compared to that in the CHG2 system. Moreover, there was a slight shift in the OH stretching peaks at 3400–3450 cm$^{-1}$, which can possibly be due to the intermolecular H-bonding interactions among different components. Thus, it can be anticipated that the ternary system incorporating $\kappa$-carrageenan, gum arabic, and nano-hydroxyapatite was successfully fabricated with abundant H-bonding interactions between them, as depicted in Scheme 1.

2.4. XRD. XRD analysis was performed to provide the vital crystallographic data concerning the generation of nanocrystals on the surface of the nanocomposite system. In the XRD spectrum of HG, prominent diffraction peaks of n-HA were observed at 2$\theta$ = 25.4, 28.9, 33.1, 40.4, etc. (Figure 3b). Gum arabic, which is predominantly amorphous in nature, shows a broad peak at 2$\theta$ = 20–30$^\circ$, thus coinciding with the peaks of n-HA. In the ternary systems, all n-HA peaks were retained; however, there was a decrease in intensity of the peaks upon incorporating $\kappa$-carrageenan. The crystallite size of HG, CHG1, CHG2, and CHG3 using the Scherrer equation was found to be 2S, 23, 19, and 14 nm, respectively. The nanocomposite, CHG3, containing the maximum amount of CG was the most amorphous in nature. The crystallite size of CHG2 was found to be the closest to that of natural bone, thus making it a promising bone implant.

2.5. Mechanical Analysis. The structural integrity of the scaffold is vital for successful implantation, which can be maintained by appropriate mechanical strength. A comparative assessment of mechanical properties revealed that the compressive modulus and strength of the ternary systems were significantly higher than those of the binary system, as shown in Figure 4a,b. The compressive strength and modulus of CHG2 were found to be 9.2 ± 1.1 and 567 ± 2.5 MPa, respectively, which are in good agreement with previously reported values in the literature. The highest mechanical strength shown by the CHG2 nanocomposite may be because of the maximum homogeneous distribution of n-HA on its surface, as revealed by TEM images.

2.6. Biomineralization. Calcium deposition in the extracellular matrix indicates osteogenic differentiation, and it is utilized as a bone regeneration marker. The apatite layer deposition on the synthesized scaffolds was examined by SBF study for 15 days (Figure 1a2–d2) and 30 days (Figure 1a3–d3). It was observed that the mineralization of the ECM was higher in CHG1, CHG2, and CHG3 as compared to the HG scaffold. The highest mineral deposition was exhibited in CHG2 after 30 days of SBF incubation, rendering maximum number of nucleation sites. It could be attributed to the surface morphology of the CHG2 nanocomposite that exhibited a comparatively rougher and porous surface, exposing more Ca and P, leading to better cell mineralization. Thus, it can be inferred that the CHG2 scaffold can instigate adequate
biomineralization, envisaging their ability to be utilized as a potent bone scaffold.

2.7. Swelling Ratio. The biological fluids that are present in the surrounding area of the implanted scaffold must be absorbed at an efficient swelling rate. Therefore, the assessment of swelling behavior of the nanocomposites is an important criterion. A greater swelling percentage of CHG2 and CHG3 was observed as compared to the HG and CHG1 nanocomposites, as revealed in Figure 5a, which favors adhesion and proliferation of cells on the scaffolds. A higher swelling index can also aid in transporting nutrients and fluid uptake from the medium. The hydrophilic nature of κ-carrageenan might be responsible for the enhanced swelling behavior of the nanocomposites.

2.8. Biodegradation. The pertinent degradation rate should be such that when the implanted site is entirely regenerated, the scaffold must degrade concurrently. The weight loss for the CHG2 and CHG3 nanocomposites was the highest compared to HG and CHG1 after 28 days, as exhibited in Figure 5b. The degradation was primarily caused by the breakdown of the glycosidic linkage present in gum arabic and κ-carrageenan. Moreover, the higher degradation rate of CHG2 and CHG3 might be due to the greater amorphous nature, as revealed from the XRD analysis, thus stimulating passive hydrolysis.\(^{46}\)

2.9. Antibacterial Activity. The antibacterial activity of the scaffold is a vital parameter, as the critically wounded bone tissue may be associated with infection, which can induce sparse blood supply in the damaged sites, impeding the restoration of bone structure.\(^{47}\) The antibacterial nature of the scaffolds was examined on Escherichia coli and Listeria monocytogenes. The inhibitory effect of HG, as shown in Figure 6, may be due to the antimicrobial nature of GA, which was reported to hamper the bacterial growth.\(^{48}\) The nanocomposites, CHG1, CHG2, and CHG3, revealed an enhancement of the bactericidal activity. It can be explained in terms of the greater surface area of these nanocomposites because of their smaller size, leading to enhanced interactions with the bacterial cell wall.

2.10. Alizarin Red Staining (ARS). The mineralization of the extracellular matrix is an important parameter, which indicates the osteogenic differentiation of cells. The quantification of deposited calcium was accomplished by staining with ARS, which forms water-insoluble salts with Ca. All scaffolds stained with ARS exhibited a red color on the surface with the lowest intensity displayed by HG and the highest intensity shown by the CHG2 nanocomposite, as revealed in Figure 7. This enhancement of the intensity of color resulted from the greater Ca content. The incorporation of κ-CG significantly enhanced the Ca deposition as CG exhibits high Ca binding affinity, which could accelerate cell differentiation and new bone formation.\(^{49}\)

2.11. Alkaline Phosphatase (ALP). ALP is a common phosphatase enzyme, which is used as the initial marker for osteogenic differentiation at the prime phase of bone tissue regeneration.\(^{50}\) In this study, osteoblastic cells were seeded on various scaffolds for 1, 3, 7, and 10 days and assessed for their ALP activity. Among the four nanocomposites, CHG2 expressed the highest ALP activity at day 7 of cell culture, as shown in Figure 8a, which may be due to the presence of rougher surface escalating cell attachment. This result was also analogous to the ARS analysis in which CHG2 exhibited the highest Ca content, indicating significant osteoblast differentiation. There was a drop in ALP activity at day 10, possibly due to the shifting of cells to the maturation phase.

2.12. Protein Adsorption. The principal phenomenon that occurs at the composite–tissue intersection following scaffold implantation is protein adsorption, which further guides the succeeding cellular behaviors.\(^{51}\) It was observed that the adsorbed protein on the ternary systems was remarkably higher than that on the binary one (Figure 8b). The incorporation of κ-CG enhanced the protein adsorption behavior of the scaffolds, and a value of 98 μg/cm\(^2\) was observed for the CHG2 nanocomposite, which may be due to the ability of CG to absorb proteins on its surface.\(^{52}\) Furthermore, the rougher surface and high surface area might also be responsible for the enhanced activity, thus making it a promising bone construct.
Figure 8. (a) ALP analysis of CHG1, CHG2, CHG3, and HG nanocomposites. (b) Protein adsorbed on CHG1, CHG2, CHG3, and HG nanocomposites. Statistical significance level by t-test (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 9. (a) Cell viability for CHG1, CHG2, CHG3, and HG nanocomposites. (b) Hemolysis percent for CHG1, CHG2, CHG3, and HG nanocomposites. Statistical significance level by t-test (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 10. Confocal laser scanning micrographs of (a) HG, (b) CHG1, (c) CHG2, and (d) CHG3 nanocomposites.
2.13. Cell Viability Assay. The viability of cells in the presence of the as-synthesized nanocomposites is indispensable for assessing their biocompatibility. Herein, the biological activity of HG, CHG1, CHG2, and CHG3 was evaluated by in vitro culturing of MG63 cells. Figure 9a reveals that the synthesized ternary nanocomposites CHG1, CHG2, and CHG3 were more cytocompatible as compared to the HG nanocomposite. However, among the ternary systems, CHG2 was found to have significantly higher cell viability. These results suggest that our fabricated nanocomposite can be envisioned as a successful host for bone cells.

2.14. Red Blood Cell (RBC) Lysis. One of the pivotal conditions for a material to be biocompatible is that it should be accepted by the host tissue without prompting any undesirable effect, such as lysing of erythrocytes. The bone healing process is greatly influenced by the interaction between the scaffold and blood. The lysis% in our synthesized nanocomposites was very less even at a higher concentration of 50 μg/mL. The ternary nanocomposites exhibited a lysis of less than 3%, and further among them, the minimal value was observed for the CHG2 scaffold (Figure 9b). The lysis data implied that the CHG2 nanocomposite can be clinically implanted owing to its minimal effect on RBC lysis, which can generate thrombogenesis.53

2.15. Cell Attachment. The quantification of the MG63 cells adhered to the nanocomposite scaffolds for 24 h was performed by confocal laser scanning microscopy. The cells were stained with DAPI staining to visualize the nucleus. In the HG nanocomposite, it was observed that there was minimum cell attachment and proliferation, as shown in Figure 10. In the CHG1 nanocomposite, cell proliferation was observed and there was an increase in the number of cells as compared to the HG scaffold. Among the ternary systems, the presence of maximum cell attachment was observed prominently in the CHG2 nanocomposite. Thus, the synthesized nanocomposite fulfilled an important prerequisite of a biocomposite to be utilized in bone regeneration procedures.54

2.16. Western Blot. The expressions of osteogenic proteins, osteocalcin, osteonectin, osteopontin, and collagen, were analyzed by western blotting against β actin as a control. Higher intensities of immunoreactive protein bands, which favors bone mineralization and remodeling, were exhibited by the CHG1, CHG2, and CHG3 ternary systems as compared to the HG nanocomposite, as shown in Figure 11. Quantitative analysis further revealed that the CHG2 nanocomposite exhibited a 2.6-, 2-, 1.8-, and 1.6-fold increase in the levels of osteocalcin, osteonectin, osteopontin, and collagen, respectively, which was significantly higher than the other nanocomposite systems. Further, the expression of osteocalcin was maximum with respect to the other proteins. The presence of these protein expressions is a marker of osteogenic differentiation and bone formation; thus, their enhanced level implicates that the CHG2 nanocomposite can be successfully employed for bone regeneration procedures.55

3. CONCLUSIONS

In pursuit of fabricating ideal bone scaffold materials, we have developed a nanocomposite system with the bioactive phase intertwined into a biodegradable polymer matrix. Ternary systems with different composition ratios of n-HA, GA, and κ-CG were synthesized using a co-precipitation method and were compared with a binary system for a relative account of favourability and biocompatibility. Morphological SEM studies revealed that among the synthesized ternary systems CHG2 exhibited the maximum apatite layer growth. Comparative assessment of morphological, physicochemical, and biological studies revealed that ternary systems possess superior biocompatibility, protein adsorption, and osteogenic protein expression as compared to the binary system HG. Furthermore, confocal studies confirmed maximum cell proliferation in the case of CHG2, demarcating it as a promising candidate for bone tissue engineering applications. Hence, it was concluded that the κ-carrageenan-incorporated ternary system surpasses the binary system collectively in terms of bioactivity with the maximal activity shown by the CHG2 nanocomposite.

4. EXPERIMENTAL SECTION

4.1. Materials. Gum arabic, κ-carrageenan, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), rabbit anti-goat horseradish peroxidase-conjugated secondary IgG, phosphate-buffered saline (PBS), and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Sigma-Aldrich and Invitrogen, respectively. Nitrocellulose membranes were purchased from Bio-Rad. Diammonium hydrogen phosphate (NH₄)₂HPO₄ (DAHP) (99%), sodium hydroxide (NaOH) (>97%), sodium chloride (NaCl), sodium bicarbonate (NaHCO₃), dimethyl sulfoxide (DMSO), ammonium solution (25%), dipotassium hydrogen phosphate trihydrate (K₂HPO₄·3H₂O), magnesium chloride hexahydrate (MgCl₂·6H₂O), sodium sulfate (Na₂SO₄), calcium chloride (CaCl₂), calcium nitrate tetrahydrate [Ca(NO₃)₂·4H₂O] (99%), potassium chloride (KCl), tris(hydroxymethyl)aminomethane (TRIS) and hydrochloric acid (HCl) were procured from Merck, Mumbai, India.

4.2. Synthesis of Nano-Hydroxyapatite. The synthesis of n-HA was performed by a co-precipitation method in which 0.4 M orthophosphoric acid was added into 0.6 M calcium hydroxideaq. solution maintaining a Ca/P ratio of 1.67. The
mixture was then stirred vigorously at 2000 rpm. Ammonia solution (25%) was added gradually to the mixture to maintain the pH above 10 throughout the reaction. Stirring was continued for 24 h, and then it was kept for 48 h for aging. The white precipitate was then filtered and neutralized by repeated rinsing with deionized water and then dried in an oven at 110 °C.56

4.3. Synthesis of Nano-Hydroxyapatite/Gum Arabic–κ-Carrageenan Nanocomposites. To synthesize the nano-hydroxyapatite/gum arabic/κ-carrageenan ternary nanocomposite system, a co-precipitation approach was adopted. Co-precipitation is a facile and convenient way for the fabrication of nanocomposites. The synthesis of n-HA/GA/κ-CG in the ratio of 60/20/20 is described, and the rest were synthesized in the same manner.56,57 A 2 wt % solution of κ-CG was prepared by dissolving it in water and continuously stirring until a clear solution was observed. Similarly, 2 wt % GA solution was also prepared in water. The blend of κ-CG and GA was formed by mixing them together with vigorous agitation until the solution was homogenized. Next, a 6 wt % suspension of nano-hydroxyapatite was formulated by ultrasonically dispersing it in double-distilled water for 4 h. This suspension was then incorporated into the κ-CG–GA mixture dropwise while stirring continuously. To adjust the pH of the mixture (~11), a 0.5 M NaOH solution was added. The stirring was continued for another 24 h, and then it was left undisturbed for 48 h, resulting in a creamish-white precipitate. After the filtration of the precipitate, it was washed several times to neutralize the pH and afterward oven dried at 80 °C. All three ternary systems, CHG1, CHG2, and CHG3, were synthesized by the same procedure. A binary system incorporating GA and n-HA was also prepared simultaneously.

4.4. Physicochemical Characterization. Several techniques were employed to analyze the various properties of the formulated nanocomposites. To determine the composition of the nanocomposites, Fourier transform infrared spectroscopy was employed with a frequency range of 4000–400 cm⁻¹. A Philips PW 1710 diffractometer with Cu Kα radiation of 1.540 A was utilized to measure the crystalline nature of the compounds in the scale of 10–80°. The microstructural investigation was done using scanning electron microscopy (SEM, JEOL-JAPAN). The particle size and homogeneity were explored by transmission electron microscopy (TEM, Hitachi H-7500 Japan) at 120 kV. The compressive strength and modulus were determined with a universal testing machine (Schimadzu with an Instron microtensile tester, 5848) having a crosshead speed of 10 mm/min.

4.5. Swelling Ratio. All nanocomposite samples were equally weighed and the initial dry weights were calculated as $W_i$. The scaffolds were then suspended in PBS at pH 7.4 and kept for 48 h at 37 °C. After withdrawing the nanocomposites, their adsorbed water was removed and weighed again as $W_c$. The swelling ratio was determined from the formula

\[
\text{swelling \% } = \left( \frac{W_c - W_i}{W_i} \right) \times 100
\]

4.6. Biomineralization Study. To determine the biomineralization ability of nanocomposites, they were immersed in 20 mL of SBF solution, which was prepared as described in an earlier report.59 The pellets soaked in SBF were incubated for 15 and 30 days at 37 °C. Thereafter, they were removed and analyzed with a scanning electron microscope to observe the formation of the apatite layer.

4.7. ARS. Alizarin Red staining (ARS) was conducted on the scaffolds to visualize the mineralization process with respect to the method reported by Pant et al.60 ARS solution of 40 mM was synthesized in deionized water with a slightly acidic pH. The undissolved particles were eliminated by storing them in the dark after filtration. After incubation of scaffolds for 10 days, they were washed and fixed with 3.7% buffer formaldehyde. These nanocomposite formulations were then stained with the previously synthesized ARS solution for 30 min, and all excess dye was removed by repeated rinsing. The bound ARS was withdrawn by treating the scaffolds with 50% acetic acid, and the absorbance was recorded at 550 nm.

4.8. Antibacterial Activity. The antibacterial activity was determined using the agar diffusion method.61 The inoculum was developed by diluting the cultures with sterile normal saline to a 0.5 Mcfarland standard. For the preparation of agar Petri plates, a mature broth culture of particular bacterial strains was spread with $1 \times 10^5$ CFU per 50 μL. The parent solution of the nanocomposites was immersed in sterile PBS and all procedures were performed using biosafety level II hoods. The Petri plates were incubated overnight at 37 °C. The antibacterial activity of the nanocomposites was examined based on the zone of clearance formed against pathogenic strains E. coli and L. monocytogenes.

4.9. ALP. Osteogenic differentiation of the osteoblast cells was assessed by alkaline phosphatase activity. In this method, dephosphorylation of p-nitrophenyl phosphate (pNpp) was done by ALP, as reported by Whyte et al.62 The culturing of cells was performed in DMEM containing 1% penicillin–streptomycin and 10% fetal bovine serum (FBS). After the cell culturing, they were seeded on the scaffolds in polystyrene six-well dishes. Cultured medium was discarded after 4 days, and subsequently, the nonadherent cells were removed by rinsing with PBS. A 5% CO₂/95% air atmosphere was created to culture the adherent cells at 37 °C with medium refreshed once every 2 days. The merged cells were segregated with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25%) and then cultured again in six-well culture dishes at 5 x 10⁶ cells/dish. The ALP activity was measured after 1, 3, 7, and 10 days of cell culture. Briefly, 0.012 M p-nitrophenyl phosphate was added to 0.05 M diethanol amine to form an active reagent, which was added to 50 μL of Triton lysate at pH 9.8 for 30 min (37 °C) and then 50 μL of stop solution (2.5 M NaOH) was added. The absorbance was calculated at 405 nm with p-nitrophenol as the standard.

4.10. Biodegradation. The biopotency of the scaffolds was also evaluated with in vitro degradation analysis.63 To investigate the biodegradability, the nanocomposites were dissolved in PBS + lysisyme for 7, 14, 21, and 28 days analogous to the circulation level of blood (10 000 U/L). The dry weight of the samples was measured as $W_i$, and then the incubated samples were cleansed with DI water, air-dried, and weighed. The weight change was calculated as $W_c$. The following equation was used for calculating degradation percentage.

\[
\text{biodegradation \% } = \left( \frac{W_i - W_c}{W_i} \right) \times 100
\]

4.11. Protein Adsorption. The quantitative assessment of the amount of protein molecules that were adsorbed on the surface of the synthesized scaffold was performed by protein adsorption assay. After washing the nanocomposites with PBS, they were allowed to dry, and then 500 μL of FBS was used for incubation at 37 °C for 2 h. The nonadherent proteins were
removed by PBS. Then, the proteins which were adsorbed on the nanocomposite surface were extracted with 1 mL of SDS aq. solution (1 wt %). The quantification of proteins was performed using a protein analysis kit (Micro BCA Protein Assay Kit, Pierce Biotechnology, IL).

4.12. MTT Assay. The cytotoxicity of the cells seeded on the scaffolds was determined by performing MTT assay on human osteoblast-like MG63 cells. Briefly, the cells were deposited on the scaffolds with a density of 0.5 × 10⁶ cells/well in a 96-well culture plate. The nanocomposite concentration was increased from 0 to 512 μg/mL. A humidified atmosphere was adopted for incubating the culture plates for 24 h at 37 °C with a CO₂ concentration of 5%. For eliminating the dead cells, washing with PBS was done after incubation. MTT solution (0.5%) was added to the adherent cells and then incubated for 4 h. The viable cells converted MTT into insoluble formazan crystals, which were then dissolved in 0.1% DMSO. The absorbance was calculated at 570 nm, and the cell viability was measured.

4.13. RBC Lysis. The in vitro erythrocyte lysis test was carried out to investigate the toxicity level produced by the synthesized scaffolds. As illustrated by Archana et al., the degree of hemolysis was evaluated by calculating the quantity of hemoglobin ejected by breaking of the erythrocyte membrane. In brief, after the extraction of blood from a healthy male rabbit into a sterile tube containing an anticoagulant (EDTA), it was centrifuged for 10 min at 1000g and a temperature of 4 °C. The buffy coat having white blood cells and platelets along with the plasma were cautiously removed. The RBCs were delicately washed with 20 mM PBS to develop 50% hematocrit. RBCs were incubated in triplicate with varying concentrations (10⁻⁵ to 10⁻² g/mL) of scaffolds at 37 °C for 1 h to measure the degree of hemolysis induced by the nanocomposites. The centrifugation of samples at 1500 g was performed using a protein analysis kit (Micro BCA Protein Assay Kit, Pierce Biotechnology, IL).

% haemolysis = \[ \frac{A(t) - A(0)}{A(100) - A(0)} \times 100 \]

where A(t) is the absorbance of the supernatant from the samples incubated with the nanocomposites, A(0) is the absorbance of the supernatant of controls (normal saline), and A(100) is the absorbance of the supernatant of controls, which were incubated in the presence of 1% Triton X-100, causing total lysis of RBCs.

4.14. Cell Attachment. The scaffolds were seeded with osteoblast cells in a standard medium at a density of 2 × 10⁶ cells/mL in a six-well culture plate for 24 h. They were then stained with 10 mg/mL DAPI at 37 °C for 10 min to visualize the nuclei. Cell imaging was done on a confocal laser scan Zeiss LSM780 microscope with a 63× Plan-Apo/1.3 NA oil immersion objective with differential interference contrast capability. The processing of images was performed by ZEN image processing software.

4.15. Western Blot. Ice-cold radio immunoprecipitation assay (RIPA) buffer (Thermo Scientific) (Product Code. 10017003) was used to extract the protein from the cell–scaffold construct. After purification, the protein lysates were concentrated. The overall protein present was quantified with BCA assay (Pierce) (Catalog number: 23225) according to the protocol described by the manufacturer. The proteins, denatured for 5 min at 90 °C, were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then shifted to nitrocellulose membranes via a semi-dry blotter (Amersham TE 77 PWR). The membranes were blocked by nonfat milk (5%) in tris-buffered saline (TBST) for 1 h. They were then incubated with primary antibodies for polyclonal rabbit anti-human β-actin (Santa Cruz) (sc-47778), osteopontin (OPN) (Santa Cruz) (sc-73631), osteocalcin (OCN) (Santa Cruz) (sc-365797), osteonectin (ON) (Santa Cruz) (sc-33645), or collagen (COL) (Santa Cruz) for 4 h. The dilution of primary antibodies was done in TBST at a diluted concentration of 1:100. After washing the membranes with TBST, they were then incubated with rabbit anti-goat horseradish peroxidase-conjugated secondary IgG (AP106P) for 1 h at 1:5000. They were then washed three times with TBST. To visualize the immunoreactive bands, a Clarity™ Western ECL Substrate (Bio-Rad) (170-5060) and a GS800 imaging system were used. The quantitative analysis of the band intensities was done using ImageJ software (https://image.nih.gov/ij/).

4.16. Statistical Analysis. Student’s t-test was used to perform the statistical comparisons taking significance level of p < 0.05. All results were in triplicate and depicted as mean ± standard deviation.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03761.

- AFM images of CHG1, CHG2, CHG3, and HG nanocomposites (Figure S1) (PDF)

AUTHOR INFORMATION
Corresponding Author
Mohammad Shakir — Inorganic Chemistry Laboratory, Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India; orcid:0000-0002-5238-411X;
Phone: +91-9837430035; Email: shakir078@yahoo.com

Authors
- Sumbul Mirza — Inorganic Chemistry Laboratory, Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India
- Reshma Jolly — Inorganic Chemistry Laboratory, Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India
- Iram Zia — Inorganic Chemistry Laboratory, Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India
- Mohd Saad Umar — Molecular Immunology Group Lab, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India; orcid:0000-0002-6761-255X
- Mohammad Owais — Molecular Immunology Group Lab, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b03761

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
The authors acknowledge the grant support from UGC, SAP (DRS-II), DST (FIST), and DST (PURSE) to the Department of Chemistry. The author, S.M., acknowledges the CSIR-SRF for financial support.
(36) Zamora-Sequeira, R.; Ardaoi, I.; Starbird, R.; García-González, C. A. Conductive Nanostructured Materials Based on Poly-(3,4-Ethlenedioxythiophene) (PEDOT) and Starch/κ-Carrageenan for Biomedical Applications. Carbohydr. Polym. 2018, 189, 304–312.

(37) Saini, R. K.; Bagri, L. P.; Bajpai, A. K. Nano-Silver Hydroxyapatite Based Antibacterial 3D Scaffolds of Gelatin / Alginate / Poly (Vinyl Alcohol) for Bone Tissue Engineering Applications. Colloids Surf., B 2019, 177, 211–218.

(38) Liu, H.; Webster, T. J. Mechanical Properties of Dispersed Ceramic Nanoparticles in Polymer Composites for Orthopedic Applications. Int. J. Nanomed. 2010, 5, 299–313.

(39) Shakir, M.; Jolly, R.; Khan, M. S.; Iram, Ne.; Khan, H. M. Nano-Hydroxyapatite/Chitosan-Starch Nanocomposite as a Novel Bone Construct: Synthesis and in Vitro Studies. Int. J. Biol. Macromol. 2015, 80, 282–292.

(40) Ibekewe, C. A.; Oyatogun, G. M.; Esan, T. A.; Olawusengo, K. M. Synthesis and Characterization of Chitosan / Gum Arabic Nano Particles for Bone Regeneration. Am. J. Mater. Sci. Eng. 2017, 5, 28–36.

(41) Kim, J. H.; Kim, D. K.; Lee, O. J.; Ju, H. W.; Lee, J. M.; Moon, B. M.; Park, H. J.; Kim, D. W.; Lee, J. H.; Park, C. H. Osteoinductive Silk Fibroin/Titanium Dioxide/Hydroxyapatite Hybrid Scaffold for Bone Tissue Engineering. Int. J. Biomol. 2016, 82, 160–167.

(42) Lowe, B.; Venkatesan, J.; Anil, S.; Shim, M. S.; Kim, S. K. Preparation and Characterization of Chitosan-Natural Nano Hydroxyapatite-Fucoidan Nanocomposites for Bone Tissue Engineering. Int. J. Biol. Macromol. 2016, 93, 1479–1487.

(43) Emam, H. E. Arabic Gum as Bio-Synthesizer for Ag – Au Bimetalllic Nano Composite Using Seed-Mediated Growth Technique and Its Biological Efficacy. J. Polym. Environ. 2018, 27, 210–223.

(44) Kim, T.; Kim, M.; Goh, T. S.; Lee, J. S.; Kim, Y. H.; Yoon, S.; Lee, C. Evaluation of Structural and Mechanical Properties of Porous Artificial Bone Scaffolds Fabricated via Advanced TBA-Based Freeze-Gel Casting Technique. Appl. Sci. 1965, 9, 1–17.

(45) Zheng, S.; Guan, Y.; Yu, H.; Huang, G.; Zheng, C. Polyl-L-Lysine-Coated PLGA/Polyl(Amino Acid)-Modified Hydroxyapatite Porous Scaffolds as Efficient Tissue Engineering Scaffolds for Cell. New J. Chem. 2019, 43, 9969–10002.

(46) Lim, J.; You, M.; Li, J.; Li, Z. Emerging Bone Tissue Engineering via Polyhydroxalkanoate (PHA)-Based Scaffolds. Mater. Sci. Eng. C 2017, 79, 917–929.

(47) Wei, P. F.; Yuan, Z.; Jing, W.; Guan, B.; Liu, Z.; Zhang, X.; Mao, J.; Chen, D.; Cai, Q.; Yang, X. Regenerating Infected Bone Defects with Osteocompatible Microspheres Possessing Antibacterial Activity. Biomater. Sci. 2018, 7, 272–286.

(48) Hindi, N. K.; Bnuyan, I.; Jebur, M.; Mahdi, M. A. In Vivo Antimicrobial Activity of Gum Arabic (Al Manna and Tayebat) Prebiotics against Infectious In Vitro Antimicrobial Activity of Gum Arabic (Al Manna and Tayebat) Prebiotics against Infectious Pathogens. Int. J. Pharm. Pharm. Res. 2015, 3, 77–85.

(49) Debon, S. J.; Tester, R. F. In Vitro Binding of Calcium, Iron and Zinc by Non-Starch Polysaccharides. Food Chem. 2001, 73, 401–410.

(50) Kerativitayanan, P.; Tatullo, M.; Khariton, M.; Joshi, P.; Selvamurugan, N.; Verwanger, T.; Mathur, S.; Beppu, M. M.; Chevallier, P.; Mantovani, D.; Vieira, R. Food Chem. 2015, 160, 1479–1485.

(51) Shakir, M.; Jolly, R.; Khan, M. S.; Iram, Ne.; Khan, H. M. Nano-Hydroxyapatite/Chitosan-Starch Nanocomposite as a Novel Bone Construct: Synthesis and in Vitro Studies. Int. J. Biol. Macromol. 2015, 80, 282–292.

(52) Lima, P. H. L.; Pereira, S. V. A.; Rabello, R. B.; Rodrigues-castellon, E.; Beppu, M. M.; Chevallier, P.; Mantovani, D.; Vieira, R. S. Blood Protein Adsorption on Sulfonated Chitosan and -Carageenan Films. Colloids Surf., B 2013, 111, 719–725.

(53) Maleki, H.; Shahbazi, M.; Montes, S.; Hosseini, S. H.; Eskandari, M. R.; Zausschirm, S.; Verwanger, T.; Mathur, S.; Milow, B.; Krammer, B.; et al. Mechanically Strong Silk-Silk Fibroin Bioaerogel: A Hybrid Sca Ff Old with Ordered Honeycomb

Micromorphology and Multiscale Porosity for Bone Regeneration. ACS Appl. Mater. Interfaces 2019, 11, 17256–17269.

(54) Woldetsadik, A. D.; Sharma, S. K.; Kapli, S.; Jagannathan, R.; Magouz, M. Hierarchically Porous Calcium Carbonate Sca Ff Olds for Bone Tissue Engineering. ACS Biomater. Sci. Eng. 2017, 3, 2457–2469.

(55) Yang, W.; Yao, C.; Cui, Z.; Luo, D.; Lee, J.; Yao, J.; Chen, C.; Kong, X. Poly (Acrylic Acid) -Regulated Synthesis of Rod-Like Calcium Carbonate Nanoparticles for Inducing the Osteogenic Differentiation of MC3T3-E1 Cells. Int. J. Mol. Sci. 2016, 17, No. 639.

(56) Shakir, M.; Zia, I.; Rehman, A.; Ullah, R. Fabrication and Characterization of Nanoengineered Biocompatible N-HA/Chitosan-Tamarind Seed Polysaccharide: Bio-Inspired Nanocomposites for Bone Tissue Engineering. Int. J. Biol. Macromol. 2018, 111, 903–916.

(57) Rane, V. A.; Kanny, K.; Abitha, V. K.; Thomas, S. Methods for Synthesis of Nanoparticles and Fabrication of Nanocomposites; Elsevier Ltd: 2018; pp 121–139.

(58) Saravanan, S.; Chawla, A.; Vairamani, M.; Sastry, T. P.; Subramanian, K. S.; Selvamurugan, N. Scaffolds Containing Chitosan, Gelatin and Graphene Oxide for Bone Tissue Regeneration in Vivo and in Vivo. Int. J. Biol. Macromol. 2017, 104, 1975–1985.

(59) Shakir, M.; Jolly, R.; Khan, M. S.; Iram, Ne.; Khan, H. M. Nano-Hydroxyapatite/Chitosan-Starch Nanocomposite as a Novel Bone Construct: Synthesis and in Vitro Studies. Int. J. Biol. Macromol. 2015, 80, 282–292.