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Preparation and characterization of chitosan-natural nano hydroxyapatite-fucoidan nanocomposites for bone tissue engineering

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ABSTRACT

Solid three dimensional (3D) composite scaffolds for bone tissue engineering were prepared using the freeze-drying method. The scaffolds were composed of chitosan, natural nano-hydroxyapatite (nHA) and fucoidan in the following combinations: chitosan, chitosan-fucoidan, chitosan-nHA, and chitosan-nHA-fucoidan. Fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), X-ray diffraction analysis (XRD), scanning electron microscopy (SEM), and optical microscopy (OM) were used to determine the physiochemical constituents and the morphology of the scaffolds. The addition of nHA into the chitosan-fucoidan composite scaffold reduced the water uptake and water retention. FT-IR analysis confirmed the presence of a phosphate group in the chitosan-nHA-fucoidan scaffold. This group is present because of the presence of nHA (isolated via alkaline hydrolysis from salmon fish bones). Microscopic results indicated that the dispersion of nHA and fucoidan in the chitosan matrix was uniform with a pore size of 10–400 μm. The composite demonstrated a suitable micro architecture for cell growth and nutrient supplementation. This compatibility was further elucidated *in vitro* using periosteum-derived mesenchymal stem cells (PMSCs). The cells demonstrated high biocompatibility and excellent mineralization for the chitosan-nHA-fucoidan scaffold. We believe that a chitosan-nHA-fucoidan composite is a promising biomaterial for the scaffold that can be used for bone tissue regeneration.

1. Introduction

Tissue engineering is an interdisciplinary field in which artificial organs are constructed using materials, cells, and growth factors. Bone is an important organ that remodels continuously during an individual's lifetime and forms the human skeletal framework. Bone tissue engineering is an important aspect of regenerative medicine and biotechnology. It provides alternative therapeutic methods to restore the function and repair damaged or degenerating bone tissue. Biomaterials with functional subunits provide advantages that promote bone tissue regeneration and facilitate the repair of damaged bone tissue. They have important applications in clinical orthopedics during reconstructive surgical procedures. Autologous transplantation is the most successful clinical strategy because it effectively unites the host bone tissue without immunogenic complications or the disease risks associated with allogeneic sources. Autologous transplantation is effective, but the supply is limited because of the medical and socioeconomic constraints of the world's aging population. Additionally, orthopedic reconstruction from trauma, tumors, congenital deformities, and injuries are at record numbers placing increased demand on bone regeneration and implant technologies [1]. Tissue engineers develop functional materials for orthopedic reconstruction that can deliver biochemical cues to cells. Understanding the biological effects of these materials is a fundamental requirement of tissue engineering by affirming their suitability and elucidating the role they play in tissue formation [2,3]. In bone tissue engineering, substitutes are engineered as scaffolds that are non-toxic, biocompatible, biodegradable in a controlled manner, and osteoconductive [4]. Various fabrication techniques are employed to ensure these requirements are satisfied. Techniques such as freeze-drying, particle leaching, electrospinning, soft lithography, and photolithography can produce suitable micro and nano architectures that influence cell adhesion, expansion,

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alignment, proliferation, and differentiation [5–8]. Nanotechnology can facilitate the design and fabrication of these scaffolds. A suitable micro environment (similar to the native extracellular matrix) can be modified to guide cell behavior towards the generation of implantable tissues [9].

Chitosan is a polysaccharide primarily composed of β-(1-4)-linked D-glucosamine and *N*-acetyl glucosamine subunits [10]. It is a partially deacetylated derivative of chitin and is readily soluble in dilute acids (pH < 6). Chitosan is biodegradable, biocompatible, and non-toxic and has a functional hydrophilic surface that promotes cell growth. It can be used to modify cell adhesion, proliferation, and differentiation. It also elicits nominal immunogenicity when used as an implant [10–20]. It is a widely studied material in bone tissue engineering and functionalized with other materials. Chitosan is a suitable cross-linker that also acts as a substrate biomaterial that can mimic the extracellular matrix glycosaminoglycans. It enhances cell adhesion, survival, and proliferation [21]. Several researchers have investigated the combination of chitosan, synthetic nano hydroxyapatite, and natural nano hydroxyapatite (nHA) for bone tissue engineering applications [22–26].

nHA is a natural mineral form of calcium apatite that chemically resembles the complex matrix of bone. It is composed of a hydroxyl and complex apatite denoted by the formula $Ca_{10}(OH)_2(PO_4)_6$. Its reconstruction requires substantial mechanical strength because of the nature of natural bone. nHA is a constituent of bone (approximately 70%) and is used to increase the structural rigidity of scaffolds, ensuring their suitability for bone tissue construction procedures. There is extensive research on the preparation of bone substitutes using nHA for biomedical applications because of the similarities of nHA to natural bone [27]. nHA is highly osteoconductive. It exhibits bone bonding capacity, offers an appropriate template structure for bone formation, and promotes cellular functioning allowing the expression of bone forming osteogenic markers [28]. Despite these properties, nHA is brittle and has limited application in loading–bearing applications. nHA is often functionalized with other polymers, such as chitosan, to provide the mechanical properties required for an implant in the reconstruction and regeneration of bone tissue [29]. nHA can be isolated from bovine sources [30,31], fish bone [32], and fish scales [33].

Fucoidan is composed of a primary linear chain of 1 → 3 linkage of α-L-fucopyranose and other saccharide units composed of xylose, galactose, mannose, and glucuronic acid [34,35]. α-D-glucopyranosyluronic acid residues can be found in *Cladosiphon okamuranus* and α-L-fucopyranosyl residues have been located in *Chorda filum*. The sulfate ester and fucose groups of fucoidan are essential units for cellular activities. This anionic polysaccharide has been targeted in tissue engineering applications. It triggers the biological activities of alkaline phosphatase and osteocalcin, which are phenotypic markers for the early stages of osteoblast differentiation. Additionally, they enhance febrile collagen matrix formation and stimulate angiogenesis *in vitro* and *in vivo* [36,37]. Fucoidan can also induce important osteogenic genes such as bone morphogenetic protein-2 (BMP-2), collagen-1, and osteocalcin. These genes are essential to the development of artificial bone because they improve bone cell adhesion and proliferation.

We hypothesized that the addition of fucoidan to functional carbonated nHA in chitosan would result in an excellent biomaterial composite suitable for bone repair and regeneration. We examined the functional role of these biomaterials and determined their biological activities for applications in bone tissue engineering. We fabricated a 3D scaffold using functionalized nHA from salmon with chitosan and fucoidan via a freeze-drying method. We investigated its role in the differentiation of periosteum derived-mesenchymal stem cells (PMSCs) for use in bone tissue engineering applications. We demonstrated the efficient differentiation of PMSCs using the chitosan-nHA-fucoidan composite.

2. Materials and methods

Chitosan (310 kDa) with 90% deacetylation was purchased from Kitto Life Co., South Korea. Fucoidan, from *Fucus vesiculosus*, was acquired from Sigma–Aldrich Co. (St. Louis, MO, USA). Natural nHA was isolated as previously described [32]. Acetic acid was obtained from Junsei chemical Co., Ltd. Stem cell expansion (SCE) medium was obtained from BioWhittaker®, (Walkersville, MD, USA). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Sigma, (St. Louis, MO, USA). Perisoteum mesenchymal stem cells (PMSCs) were obtained from Pusan National University Hospital. All other reagents employed in these experiments were of analytical grade.

2.1. Scaffold fabrication procedure

2.1.1. Preparation of the chitosan scaffold

A 2.5% chitosan solution was stirred at 1200 rpm overnight. The solution (5–6 g) was transferred to a Petri dish and frozen at −24 °C. The material was lyophilized using a freeze dryer. The scaffold was resuspended in 10% NaOH and flushed with an excess water to obtain a neutral pH of 7. It was then lyophilized.

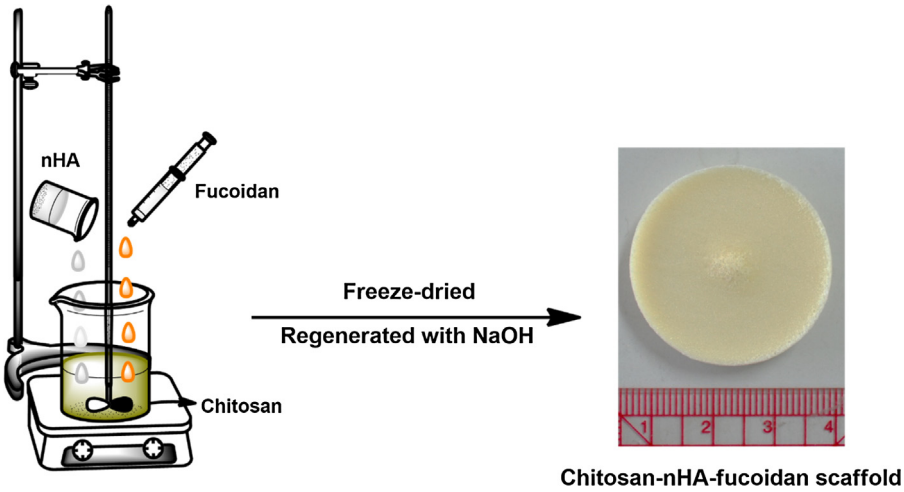

Fig. 1. Schematic representation of the fabrication method of chitosan-nHA-fucoidan scaffold.