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Three-Dimensional Composite of Fish Collagen Peptides and Bacterial Cellulose Promotes HT-22 in vitro neurite outgrowth

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ABSTRACT

Background: Bacterial cellulose (BC) is a highly biocompatible biopolymer valued for its unique nanofibrillar structure, excellent mechanical strength, high water retention, and intrinsic non-toxicity, making it particularly suitable for biomedical applications.

Methods: A three-dimensional (3D) composite scaffold composed of BC and fish collagen peptides (FCP) was fabricated via a one-step in situ biosynthesis method by incorporating optimized concentrations of FCP (0.1% and 0.5%) into the BC culture medium during bacterial fermentation. The structural integrity and surface morphology of the scaffolds were examined using field emission scanning electron microscopy (FE-SEM). The biological functionality of the scaffolds was further evaluated by culturing HT-22 neuronal cells (mouse hippocampal origin) on both pristine BC and BC-FCP scaffolds. Cell adhesion, morphology, and neurite outgrowth were evaluated using fluorescence microscopy after 2 days of incubation.

Results: The BC- FCP composite scaffolds demonstrated superior microstructural integrity and biological performance compared to pristine BC. Field- emission scanning electron microscopy revealed a denser and more uniform nanofibrous architecture in BC- FCP scaffolds, confirming the successful incorporation and uniform distribution of collagen peptides within the cellulose matrix. HT-22 neuronal cells cultured on these scaffolds showed markedly enhanced adhesion, spreading, and neurite outgrowth, particularly at the higher FCP concentrations (0.5%) demonstrating the neuro-supportive capability of the composite system.

Conclusion: The BC-FCP composite scaffolds significantly enhance neuronal adhesion and growth, making them promising candidates for neural tissue engineering and regenerative applications.

INTRODUCTION

Nerve injuries can result from various causes including accidents, sports, tumors, diabetes, Guillain–Barré syndrome, and autoimmune diseases [1, 2]. In severe nerve injuries, regeneration is challenging and often leads to partial or permanent loss of function. Therefore, as science has progressed, research has been directed toward developing effective strategies to promote nerve regeneration. However, the current methods of pharmacological interventions and surgical techniques such as allografts, xenografts, and autografts have various limitations due to limited donor availability, immune rejection, and unsuitability for extensive nerve damage [3, 4]. Currently, the development and application of biocompatible artificial scaffolds are considered a promising strategy to enhance neuronal cell growth and function through tissue engineering approaches. Various approaches such as electrospinning, bioprinting, gas foaming, and salt leaching have been used to produce artificial and natural scaffolds that can mimic the structure and function of the extracellular matrix (ECM) [5]. The ECM is primarily composed of collagen and other fibrous proteins. Due to their biocompatibility and 3D morphology, scaffolds made from biomaterials can provide a biomimetic environment for the cultured cells and promote cell adhesion, proliferation, and differentiation [6].

Various biomaterials that could support the growth of cells and tissues in 3D patterns have attracted considerable attention in tissue engineering, drug development, and organ repair, as they appear to provide more physiologically relevant and predictive in- vitro models for understanding in- vivo behavior. Among the available biomaterials, bacterial cellulose (BC) is one of the most attractive polymeric hydrogels in terms of purity and elasticity, in comparison to other sources such as plants, algae [7] and in cell-free systems [8], BC has demonstrated broad potential for biomedical applications, including tissue engineering and wound healing, due to its unique combination of biocompatibility, non- toxicity, high water- holding capacity, crystallinity, chemical modifiability, and moldability into 3D conformations [9– 12]. The nanofibrillar 3D network of BC provides an ideal microenvironment for cell adhesion, proliferation, and differentiation, while its highly porous hydrogen- bonded structure facilitates composite formation with various functional materials, further enhancing its biological and mechanical performance [7].

Aligned fibrous matrices have been extensively investigated for neuronal regeneration, as their oriented architecture provides topographical cues for axon guidance and promote neurogenic differentiation of various stem cell lineages [13]. Similarly, neurite outgrowth could be promoted by aligned fibrillar structures [14]. Furthermore, biocompatible and biodegradable aligned scaffolds have been increasingly utilized across various tissue regeneration applications, highlighting their effectiveness in enhancing cellular organization and tissue healing [15]. Beyond their structural benefits, fibrillar matrices can be further functionalized with biochemical agents including bioactive peptides, therapeutic drugs, and growth factors to enhance the biological performance of engineered scaffolds [7, 16]. Nevertheless, targeted delivery and continuous stimulation by bioactive molecules remain a major challenge in the rational design of scaffolds for tissue regeneration. Biomaterial scaffolds such as core- shell materials also provide a more suitable environment for the controlled release of encapsulated biomolecules in comparison to single-layers PCL and tri-layers PVA/PCL/PVA, electrospinning nano sheet etc. [17].

In general, nerve tissue regeneration in two- dimensional (2D) cell culture systems proceeds more slowly than in vivo, as such environments lack the three-dimensional (3D) structural and biochemical cues essential for neural growth. Therefore, 3D scaffolds designed for nerve tissue engineering must exhibit long- term biodegradability to support sustained culture and tissue maturation. Fish collagen peptides (FCP) have been shown to enhance in-vitro neurite outgrowth by promoting cellular adhesion and growth- factor signaling, significantly accelerating neurite extension in cultured neuronal cells and highlighting their potential in neural regeneration [18]. Based on this evidence, it was hypothesized that the developed 3D cell culture model based on natural BC- FCP biopolymers would be biocompatible and create a suitable microenvironment for neurite adhesion, proliferation and outgrowth.

While pure BC possesses high mechanical strength but limited cell adhesion and proliferation, combining BC with FCP enhances neuronal cell adhesion, proliferation, and neurite outgrowth. Ideal scaffolds for neuronal cell studies need these key properties, especially for in vitro cultures and various biomedical applications. This strong cell–scaffold interactions observed in BC–FCP composites are expected to promote extensive neurite network formation, particularly in

hippocampal neuronal cells.

In the current study, the fabricated BC– FCP matrices were examined for numerous organizational and biological features to explore the cell- scaffold interaction. The currently designed bioengineered composite will potentially provide a more accurate perception of cell-cell and cell-matrix interaction in neurite outgrowth. Importantly, the single-step preparation of BC– FCP composites offers a simple and scalable fabrication approach highly desirable for biomedical applications. The BC–FCP composite exhibits a nanofibrous network resembling the native ECM, excellent biocompatibility, and enhanced biofunctionality through the incorporation of FCP. The aim of the current study is to develop a biocompatible 3D cell culture model based on natural BC- FCP biopolymers to enhance the biocompatibility of neural cells and create a suitable microenvironment for neurite adhesion, proliferation and differentiation.

METHODS

HT-22 (mouse hippocampal neuronal) cells were received from the Sigma- Aldrich (SCC129) (Merck KGaA Frankfurter Str. 250 D-64271 DARMSTADT). HT-22 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and ampicillin/ streptomycin antibiotics, all obtained from Gibco Life Technologies (Grand Island, NY, USA). Fish collagen peptides (FCP) were obtained from a Chinese company (9F, New Caohejing Building, No. 509 Caobao Rd, Shanghai China). F-actin stain (FITC- phalloidin), and rest of the chemicals were obtained from Sigma Aldrich (St. Louis, USA).

Production of BC

BC sheets were prepared according to the static cultivation protocol previously reported [6]. The culturing Hestrin-Shrramm medium was sterilized by autoclaving at 121°C for 15 min at 15 psi before inoculation of the bacteria. The produced BC pellicles were collected and cleaned first by washing thoroughly with distilled water and then with 0.3 M NaOH solution for 2 to 3 hours at room temperature to remove cell debris. The NaOH solution was removed and the BC pellicles washed with distilled water three times each 10 to 15 minutes each. The BC pellicles were stored in distilled water and placed in the refrigerator for later use.

Preparation of BC-FCP scaffolds

The BC-FCP composites were fabricated (Figure 1.) by adding FCP type I (0.1% w/v and 0.5% w/v) to each flask containing *Gluconacetobacter xylinum* culture broth and incubated for about one 10 days at 30°C [6]. The composites were harvested and cleaned with distilled water and 0.3 M NaOH solution to eliminate the remaining media components and cellular debris and re-washed with distilled water. The composites were freeze- dried for two days and stored for future experiments.

FE-SEM analysis of BC and BC-FCP scaffolds

BC and BC-FCP composite samples were characterized using field emission scanning electron microscopy (FE- SEM, Hitachi S-4800, Tokyo, Japan) equipped with energy- dispersive X- ray spectroscopy (EDX-350, Horiba, Tokyo, Japan). Prior to imaging, samples were mounted on brass stubs using carbon tape and coated with a thin layer of osmium tetroxide (OsO_4) using a VD HPC- ISW osmium plasma coater (Tokyo, Japan) to enhance conductivity and image resolution. Fiber diameters were quantified using ImageJ (NIH, USA) by calibrating the SEM micrographs with the corresponding scale bar. A minimum of 50 randomly selected fibers were measured for each sample to obtain the average diameter and distribution.

Culturing of HT-22 cells on BC and BC-FCP composite and cellular adhesion analysis

HT-22 cells were cultured in complete RPMI-1640 medium containing 10% FBS in $1x10^5$ /cells per well in a 24 well plate and incubated at 37°C for 24h before seeding them on the samples. Afterward, the cultured cells were trypsinized and seeded on the surface of deionized sterilized BC and BC- FCP composites. Cell culture medium was refreshed after every 24 h. The adhesion of HT-22 cells on BC and BC-FCP were analyzed by using a microscope to count the adhere cells after 2 to 3 hours of culture and washing with physiological solution.

Cell viability assay

HT-22 Cells were seeded at $1x10^4$/cells per well in a 96 well plate on both pure BC and BC-FCP for 24 to 72 h. Cell viability was monitored after confluent culture using a WST-1 colorimetric conversion assay (EZ- Cytox assay kit, Daeil Lab Service, Seoul, Korea) according to the guidelines provided [11].

Immunocytochemistry neurite outgrowth analysis

To conduct cellular morphology and neurite outgrowth assay of the cells cultured on BC and BC-FCP composite, HT-22 cells were stained with F- actin staining solution (100– 200 nM concentration) and observed under fluorescence microscope (FV1000- IX81, Olympus, Japan) [20]. The F-actin staining was used to analyze cellular differentiation because changes in actin structure and distribution are involved in many cellular processes, such as differentiation, proliferation and migration.

Statistical analysis

Experiments were performed in at least triplicate sets and the data was presented as mean ± standard deviation (SD). Statistical significance among multiple groups (Control, BC–FCP 0.1%, and BC–FCP 0.5%) was analyzed using one-way analysis of variance (ANOVA). A *p*-values <0.05 were considered to be statistically significant.

RESULTS

Structural morphology of BC and BC-FCP scaffolds

The surface and structural characteristics of the developed BC and BC–FCP composites were examined using SEM to evaluate the effect of FCP incorporation on the native BC architecture. SEM analysis (Figure 2) revealed that pure BC retained its typical porous 3D fibrous morphology, while the BC-FCP composites exhibited compact structures, indicative of pore closure due to FCP inclusion. The diameter of BC fibers ranged from 0.03 to 0.09 µm, primarily between 0.04 and 0.06 µm. For BC-FCP (0.1%), the range was 0.042–0.104 µm and for BC-FCP (0.5%) it was 0.049– 0.095 µm, indicating a consistent and slightly broadened morphology with FCP impregnation. We have presented only the 0.1% and 0.5% groups, which exhibited more pronounced differences in scaffold properties and biological responses.

Effect of FCP on BC production, cellular adhesion and proliferation

The dry weight of BC pellicles increased noticeably with the incorporation of FCP in the culture medium (Fig. 3A). The control BC exhibited a yield of approximately 5.8 g/ L, whereas supplementation with 0.1% and 0.5% FCP enhanced the production to about 9.0 g/L and 9.3 g/L, respectively. The improvement in BC yield can be attributed to the presence of collagen peptides, which likely served as additional carbon/ nitrogen sources and biostimulants, promoting bacterial metabolism and cellulose synthesis. HT-22 hippocampal neuronal cells adhered more effectively to BC- FCP scaffolds compared to pure BC (control group), with significant improvements observed at 0.1% and 0.5% FCP concentrations (Figure 3B). WST-1 assays (Figure 3C) demonstrated that although pure BC supported higher initial cell proliferation, BC-FCP composites maintained prolonged proliferative responses over 72 hours.

Cellular morphology and Neurite outgrowth

Fluorescence microscopy (Figure 4) revealed that HT-22 cells exhibited restricted growth and adherence on pure BC. In contrast, BC- FCP scaffolds enabled greater cellular adhesion and supported guided neurite outgrowth. Cells cultured on BC- FCP scaffolds demonstrated enhanced neurite outgrowth compared to pure BC, indicating that incorporation of fish collagen peptides improved the neuro-supportive characteristics of the scaffold

Figures

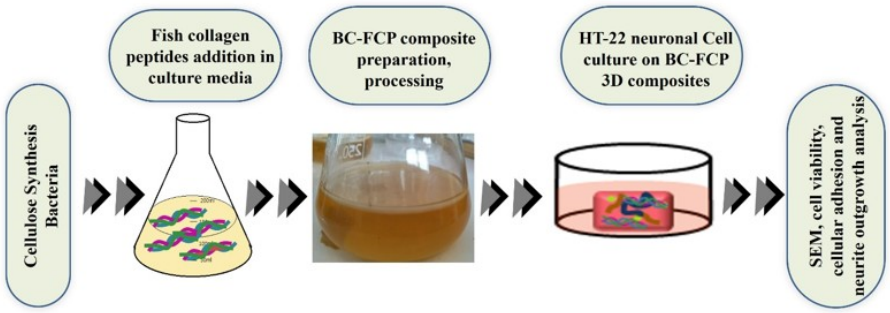

Figure 1: Graphical representation of the preparation and characterization steps for BC and the BC-FCP composite. The figure outlines the fabrication process, incorporation of FCP, and subsequent analytical methods used to evaluate the composite's properties.

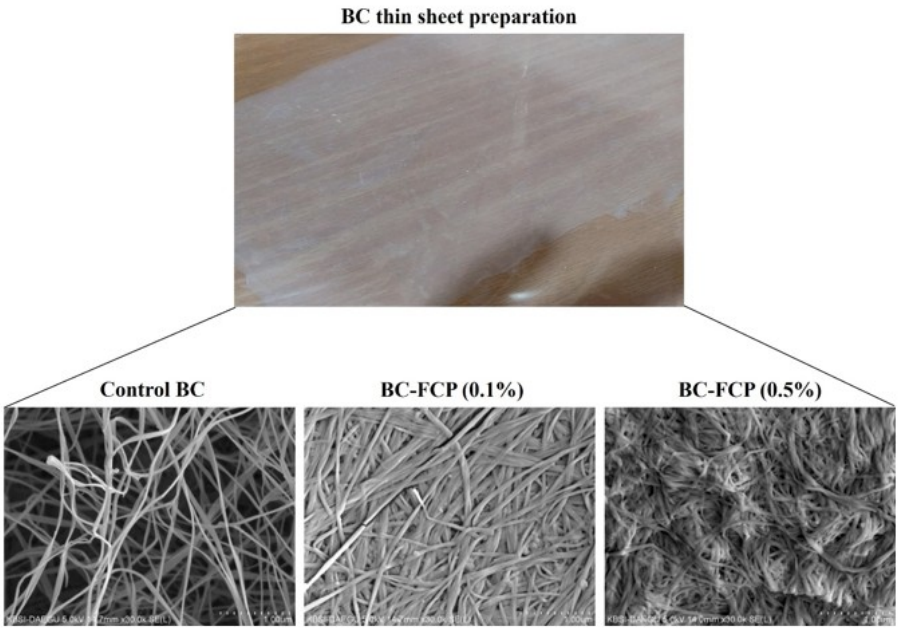

Figure 2: Schematic illustration and SEM structure analysis of FCP incorporated into the BC microfibrous network. The figure compares the structure of pure BC microfibers with the BC-FCP composite, highlighting the uniform distribution of collagen peptides both on the surface and within the internal structure of the BC matrix.

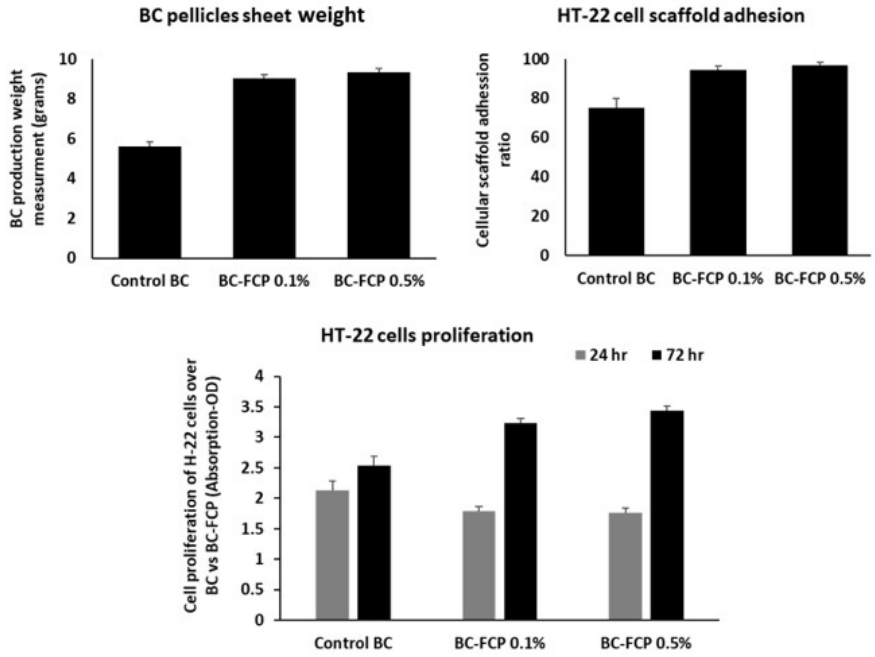

Figure 3: (A) Effect of FCP on BC weight (grams) production. (B) HT-22 cell adhesion and proliferation on pure BC and BC-FCP composites over 24, and 72 hours. (C) Cell proliferation analysis of HT-22 cells culture over BC and BC-FCP scaffolds. Statistical analysis was performed using ANOVA (p < 0.05).

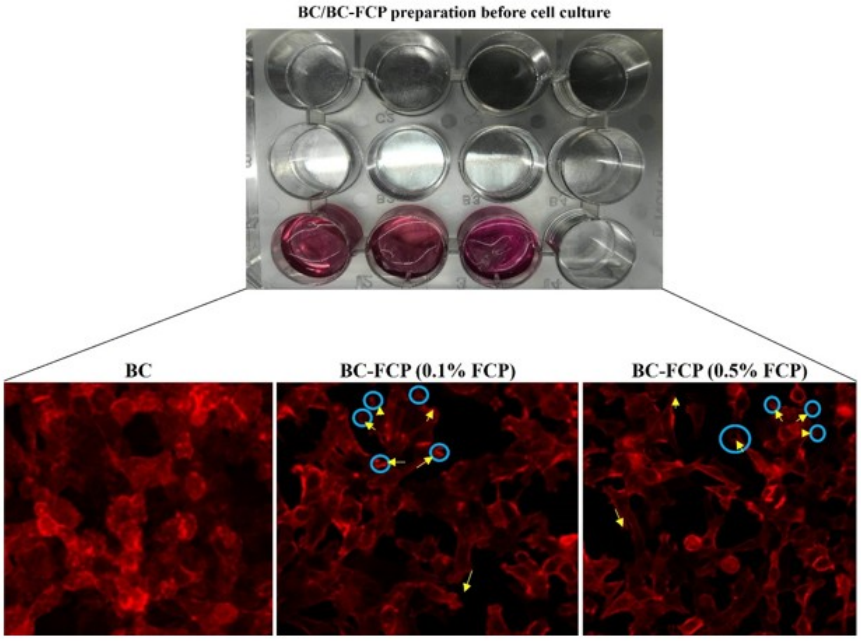

Figure 4: Fluorescence imaging of HT-22 cells cultured on pure BC and BC-FCP scaffolds (200× magnification). Pure BC exhibits stronger F-actin staining due to its denser and more restrictive morphology. In contrast, the BC-FCP composite provides a more favorable microenvironment, enhancing cellular adhesion and promoting neurite outgrowth. Compared to control BC, the biofunctional cues of the BC-FCP scaffold facilitate guided neurite outgrowth. Growth cones of HT-22 cells are indicated by blue circles, and the direction of microfilament extension is marked with yellow arrows.

DISCUSSION

SEM images confirm that FCP integration significantly alters the structural morphology of BC, increasing fiber compactness and narrowing pore sizes yet preserving the scaffold's integrity as reported earlier [21]. The observed uniformity in fiber diameter distribution suggests enhanced structural consistency favorable for tissue engineering applications. The improved interaction between FCP and BC fibers can be attributed to hydrogen bonding, consistent with earlier studies on BC composites [7].

Increased BC production upon FCP incorporation suggests a stimulatory effect on *Gluconacetobacter xylinum* , potentially due to improved nutrient bioavailability or enhanced microbial metabolism mechanisms that merit further exploration. As seen in Figure 3, the dose-dependent increase in BC yield reinforces this hypothesis. Cellular adhesion assays demonstrate that low molecular weight collagen peptides effectively promote HT-22 cell binding, aligning with previous work showing their superior bioactivity [11, 13].

Interestingly, while BC alone supports high short- term proliferation, FCP- functionalized BC maintains a more stable long- term cell viability. This indicates that the biophysical and biochemical cues imparted by FCP enhance cell-scaffold interactions over time.

The fluorescence imaging in Figure 4 supports the notion that BC- FCP scaffolds provide an improved microenvironment for neuronal differentiation and neurite outgrowth. Enhanced cellular adhesion and the prominent presence of growth cones indicate active and extended neurite projection, which appeared more pronounced than on pure BC scaffolds. The incorporation of FCP into the BC matrix likely improved surface bioactivity, providing biochemical cues and structural flexibility favorable for neurite elongation and network formation. Such enhanced neurite spreading and interconnectivity on composite scaffolds are consistent with previous findings on functionalized biomaterials that promote neuronal growth [22–25].

Nonetheless, the inclusion of bioactive ligands collagen peptides within the BC matrix offers key advantages. These peptides support not only adhesion and proliferation but may also serve as biochemical anchors for future biofunctionalization strategies. Literature suggests that such ligand- mediated interactions are pivotal in promoting neuronal responses on biomaterial scaffolds [23, 26]. Furthermore, the mechanical robustness of BC and the bioactivity introduced by FCP create a promising platform for long-term applications in neural tissue engineering.

The present study demonstrates a novel strategy for preparing BC- FCP biocomposites by adding an optimized concentration of FCP prior to the accumulation of BC pellicles in a bacterial cell culture. This method is designed to enhance neuron adhesion and growth. The newly synthesized BC- FCP composites provide both topographical and biochemical cues for neurogenesis in an in vitro 3D culture. Our 3D cell experiments have shown that BC- FCP composites possess the structural, physicochemical, and biological compatibility needed for use as tissue engineering scaffolds in neuronal studies. The modified surface of BC- FCP facilitates neurite adhesion and growth by providing bioactive cues. Our results indicate that these modified BC- FCP composites are highly promising for accelerating the adhesion and differentiation of neuronal cell cultures and promoting the regeneration of neuronal tissue. We believe that integrating FCP during the formation of BC pellicles offers a new opportunity for advancing research in neuronal cell molecular biology and the biomedical applications of cellulose-based functional materials.

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CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Writing review and editing: Fanar Hamad Alshammari, Mazhar Ul-Islam, and Fazli Subhan.

Supervision: Mazhar Ul-Islam and Fazli Subhan.

Isfahan Tauseef and Mazhar Ul-Islam contributed equally to this work

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Generative AI Statement

The authors declare that Generative AI tools were used to enhance the language clarity of this work. We take full responsibility for the accuracy and integrity of the content.

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