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Beneficial Effects of Microwave-Induced Argon Plasma Treatment on Cellular Behaviors of Articular Chondrocytes Onto Nanofibrous Silk Fibroin Mesh

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Abstract: Silk fibroin scaffolds were examined as a biomaterial option for tissue-engineered cartilage-like tissue. In tissue engineering for cartilage repair using a scaffold, initial chondrocyte-material interactions are important for the following cell behaviors. In this study, the surface of nanofibrous silk fibroin (NSF) meshes was modified by a microwave-induced argon plasma treatment in order to improve the cytocompatibility of the meshes used as cartilaginous grafts. In addition, the effects of a plasma treatment on the cellular behavior of chondrocytes on NSF were examined. The plasma treatment resulted in an increase in the hydrophilicity of NSF meshes suggesting that the cytocompatibility of the mesh might be improved. Furthermore, the human articular chondrocytes showed higher viability on the surface-modified NSF meshes. These results suggest that the surface modification of NSF meshes by plasma can enhance the cellular behavior of chondrocytes and may be used in tissue engineering.

Keywords: nanofibrous silk fibroin, chondrocytes, cartilage, microwave-induced argon plasma, cytocompatibility.

Introduction

It is well known that adhesion and proliferation of cells to biomaterials are highly dependent on the surface properties of the substrate including cleanliness, surface charge, surface free energy and density, and nature of the polar groups.[1-3] In general, the attachment of cells to a substrate increases with increase in substrate surface free energy. Thus, effective chemical modification of a polymeric surface can significantly promote biological activities and improve cell compatibility. Modification of the chemical-group functionality, surface charge, hydrophilicity and wettability of a polymeric surface can be achieved by various chemical or physical processes, such as plasma treatment, electric discharge, surface grafting, chemical reaction, vapor deposition of metals, and flame treatment.[4,5]

Plasma treatment is a unique and versatile method for the modifying surfaces without altering bulk properties. It is commonly used to increase the surface oxygen concentration and enhance interfacial adhesion, and has been used to enhance surface wettability through introducing specific surface functionalities (e.g., amine, acid and hydroxyl groups)

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for medical applications.[6] Argon plasma has been used in many applications, such as pretreatment for grafting[7] or crosslinking[8] of surface macromolecules, removal of contaminants on the surface, and ablation of material from the surface to remove a weak boundary layer and increase surface roughness for better adhesion.

Silk fibroin (SF) is known as a potential candidate material for biomedical applications because it has several attractive properties, including good biocompatibility, good oxygen and water vapor permeability, and biodegradability. Therefore, a matrix made of SF fibers has been known to support the attachment, growth and differentiation of adult human progenitor bone marrow stromal cells.[9] In addition, SF provides a remarkable combination of strength and toughness to provide enough stability. Therefore, it may have the additional advantages of space maintenance for bone ingrowth while preventing membrane collapse.

For clinical tissue engineering applications, it is unclear what role microwave-induced argon plasma treatment has in modifying substrate properties and whether surface hydrophilicity, protein adsorption, or the bulk properties of the substrate are responsible for eliciting different adhesion and phenotypic responses in human chondrocytes. In this study, the surface of nanofibrous SF (NSF) meshes was modified by microwave-induced argon plasma treatment in order to improve their cytocompatibility and the ability to support the attachment and proliferation of chondrocytes for possible applications.

Experimental

Fabrication of NSF Meshes. SF was obtained from *Bombyx mori* cocoons by soap-soda degumming as previously described.[10] NSF was fabricated by electrospinning. Dope was prepared by dissolving the SF in 98% formic acid for 4 h and the concentration was 12%. Impurities and bubbles in the solution were removed by vacuum filtration (3~4 s). For electrospinning, the dope was put into a 10 mL syringe with a 22 G stainless steel syringe needle connected to a high voltage power supply (CPS-60 k02v1, Chungpa EMT Co., Ltd., Seoul, Korea). The dope flow rate was accurately controlled by a metering pump (KD Scientific Inc., MA, USA). A grounded rolling metal drum was used as a collector for sheet-like NSF mesh fabrication. The electrospinning was carried out at room temperature and 60% humidity. Electric potential and distance to collector were fixed at 12 kV and 10 cm, respectively. After electrospinning, the NSF sheet was separated from the drum, immersed in methanol for 1 h for insolubilization and re-crystallization of SF, and then dried for 2 wk under tension in order to prevent shrinkage. Afterwards, it was cross-linked with glutaraldehyde vapor in a sealed chamber for 1 d and immersed in 0.1 M glycine in 0.2 M sodium carbonate buffer (pH 9.2) for 1 d to neutralize toxicity of glutaraldehyde. After several washes in phosphate-buffered saline, the product was lyophilized at 40 °C again. The final product was considered as a NSF mesh and cut into a disk (9 mm in diameter and 0.15 mm in thickness).

Surface Modification of NSF Mesh by Microwave-Induced Argon Plasma. As previously described,[11,12] NSF meshes were treated with a 2.45 GHz, waveguide-based, microwave-induced argon plasma system at atmospheric pressure for 6.2 and 12.4 s. This system consists of a 1 kW magnetron power supply, a WR-284 copper waveguide and an applicator including a tuning and a nozzle section. Argon was used as a working gas for this plasma system, and the gas flow rate is approximately 100 μL/min at 0.6 kgf/cm^2.

Characterizations of NSF Meshes.

Water Contact Angle Measurement: In order to examine the effects of plasma treatment on the hydrophilicity of NSF meshes, the mesh surface was characterized by static water contact angle measurements using the sessile drop method. For the sessile drop measurement, a water droplet of approximately 10 μL was placed on the dry surface of each mesh. The contact angle of water onto the mesh was detected at room temperature using a SEO contact angle analyzer (Phoenix 300A, Surface Electro Optics Co. Ltd., Suwon, Korea) equipped with a special optical system (Image X ver 5.0, Surface Electro Optics Co. Ltd., Suwon, Korea) and a charge-coupled device camera (SSC–DC10, Sony Co., Tokoy, Japan).

Fourier Transform Infrared Spectroscopy (FTIR): The surface chemical composition of NSF meshes treated without or with microwave-induced argon plasma was investigated by a Fourier transform infrared spectroscopy (Bruker IFS-66/S, Billerica, Massachusetts, Germany). Polarized absorbance spectra of fibrils were recorded using a Bruker IFS 66/s spectrometer in the spectroscopic range between 600 and 4000 cm^{-1} with a resolution of 0.1 cm^{-1}. The measurements were carried out at room temperature.

Scanning Electron Microscopy (SEM): The surface morphology of NSF meshes treated without or with microwave-induced argon plasma was observed under a scanning electron microscope (Hitachi S-800, Tokyo, Japan). The meshes were mounted and sputter-coated with gold/platinum using an ion coater (E1010, Hitachi) and then observed at an accelerating voltage of 20 kV.

Cell Cultures and Conditions. Neonatal human knee articular chondrocytes (nHAC-kn, Lonza, Walkersville, MD) were cultured in chondrocyte basal medium (Lonza) supplemented with 5% fetal bovine serum (Lonza), chondrocyte growth factors (Lonza, 1 mL R3-IGF-1, 2.5 mL bFGF, 1 mL insulin, 0.5 mL GA-1000 and 0.5 mL transferrin per 500 mL) and a 1% antibiotic antimycotic solution (including 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL, Sigma-Aldrich Co., St. Louis, MO) at 37 °C and 5% CO_2 in a humid environment. In order to examine the effects of plasma treatment on the cellular behaviors of

nHAC-kn, the cells were seeded onto NSF meshes treated without or with plasma and incubated for 4 h (with an initial cell density of 1.0×10^5 cells per a mesh for attachment assay) and 1, 3 and 5 d (with an initial cell density of 6.0×10^3 cells per a mesh for proliferation assay).

Cell Attachment and Proliferation Assays. MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product] was used to estimate cell attachment and proliferation onto NSF meshes according to plasma treatment time. nHAC-kn cultured onto NSF meshes treated without or with plasma were incubated with 0.5 mg/mL of MTT in the last 4 h of the culture period tested at 37 °C in the dark. The media were decanted and then washed twice with phosphate-buffered saline. The produced formazan salts were dissolved with dimethylsulphoxide, and the absorbance was determined at 570 nm by an ELISA reader (SpectraMax 340, Molecular Device, Sunnyvale, CA).

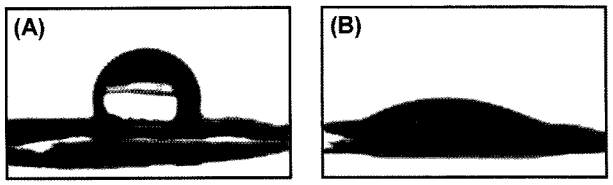
Additionally, the morphologies of cells grown onto the non-treated or plasma-treated NSF meshes were observed after 5 d of incubation by SEM. In brief, the meshes were washed with 0.1 M cacodylate buffer (pH 7.4) to remove unattached cells. The cells were fixed with 2.5% glutaraldehyde solution overnight at 4 °C, dehydrated with a series of increasing concentration of ethanol solution and then vacuum-dried. The meshes were mounted and then observed under a scanning electron microscope (Hitachi S-800) as mentioned above.

Biochemical Measurement of Collagen Types II. The collagen proteins of the cell/gel construct were solubilized and quantified in ELISA according to the protocol of human Type II Collagen Detection Kit (Chondrex, Redmound, WA). The cell/gel construct was dissolved in 10 mg/mL pepsin/0.05 M acetic acid at 4 °C for 48 h and then in 1 mg/mL pancreatic elastase/1×TSB at 4 °C overnight. In the mixture, the collagen proteins were captured by polyclonal anti-human type II collagen antibodies and detected by biotinylated counterparts and streptavidin peroxidase. OPD (o-phenylenediamine) and H_2O_2 were added to the mixture and the spectrophotometric absorbance of the mixture was measured at a wavelength of 490 nm.

Statistical Analysis. All variables were tested in triplicate for each experiment, which was repeated twice (n=6). Quantitative data were expressed as mean ± standard deviation. Statistical comparisons were carried out with a Student's *t*-test. A value of $p < 0.05$, $p < 0.1$ was considered statistically significant.

Results and Discussion

Hydrophilicity of NSF Meshes. The water contact angle was measured to determine the effect of microwave-induced argon plasma treatment on the hydrophilicity of NSF meshes.[13] As shown in Figure 1, the hydrophilicity of NSF meshes



NSF meshes	Water contact angle (°)
non-treated	94 ± 4
plasma-treated	26 ± 5*

Figure 1. Water contact angles of NSF meshes treated with microwave-induced argon plasma for 6.2 s, measured by the sessile droplet method (*$p < 0.05$ vs. the non-treated, analyzed by a Student's *t*-test, n = 6).

was increased by plasma treatment. The water contact angle of the meshes before plasma treatment was 94 ± 4°, which was significantly ($p < 0.05$) decreased to 26 ± 5° after 6.2 s of plasma treatment. Interestingly, the value was decreased near 0° after 12.4 s (data not shown here). These results suggest that plasma treatment can enhance the hydrophilicity of NSF meshes through essentially modifying their surface. It is well-known that the surface hydrophilicity of a polymer scaffold or mesh plays an important role in homogeneous cell seeding and predominant cell growth onto it.[14] In our previous report, it has already been shown that microwave-induced argon plasma treatment provided remarkably enhanced cell affinity for the surface of *b*-glucan-grafted poly(lactic-*co*-glycolic acid) mesh.[15]

Surface Chemical Composition of NSF Meshes. Figure 2 shows the FTIR spectra of the NSF meshes after the plasma treatment. The FTIR spectra of the NSF meshes after the plasma treatment showed peaks in the region of 3500-900 cm^{-1}. After the plasma treatment, hydroxyl group

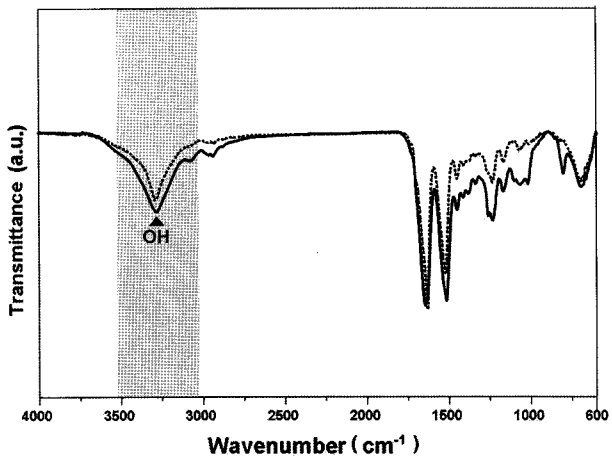
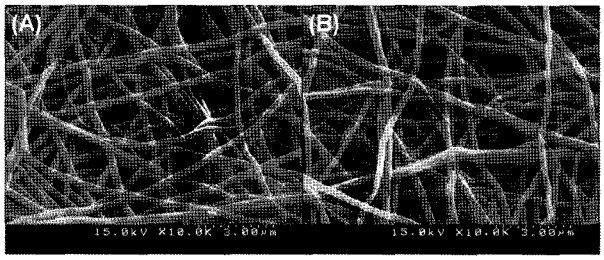


Figure 2. FTIR spectra of NSF meshes treated without [---] or with [——] microwave-induced argon plasma for 6.2 s.



Fiber diameter (nm)	Pore size (μm)	Porosity (%)
265 ± 40	1.0 ~ 2.5	73 ± 1

Figure 3. SEM micrographs of NSF meshes treated without (A) or with (B) microwave-induced argon plasma for 6.2 s (*$p < 0.05$ vs. the non-treated, analyzed by a Student's *t*-test, n = 6).

(3320-3250 cm^{-1}) content on the NSF meshes surface increased at 6.2 s. Therefore, microwave-induced argon plasma treatment creates additional hydroxyl functional groups on the NSF meshes surface. This suggests that the hydrophilicity of the NSF meshs was increased greatly by the microwave-induced argon plasma treatment.

Surface Morphology of NSF Meshes. The surface morphologies of the non-treated and plasma-treated NSF meshes were then observed by SEM (Figures 3(A) and 3(B)). It was revealed that plasma treatment did not adversely affect the surface morphology of the mesh. The plasma-treated meshes were shown to have almost similar morphology to that of the non-treated meshes. Moreover, the meshes were found to consist of entangled and randomly oriented nanofibers of about 265 nm in diameter. This morphology was similar to the fine fiber structure of natural extracellular matrix, such as collagen. The pore size of the mesh was 1.0~2.5 m and its porosity was approximately 73%.

Effects of Plasma Treatment on Cellular Behaviors of nHAC-kn onto NSF Meshes. SF as naturally occurring degradable fibrous proteins with unique mechanical properties, excellent biocompatibility and processability have been identified as a suitable scaffold material for skeletal tissue engineering.[16] To examine the effects of plasma treatment on the cellular behaviors of nHAC-kn onto NSF meshes, cell attachment and proliferation were determined according to the treatment time (Figure 4). It was found that the attachment of the cells onto the meshes was increased by plasma treatment (Figure 4(A)). The value after 12.4 s of treatment was approximately 1.5 times as high as that before treatment. It was also revealed that the proliferation of nHAC-kn appreciably increased, regardless of plasma treatment, with the progress of the incubation time (Figure 4(B)). However, the cell proliferation onto the plasma-treated meshes rather decreased as increase in plasma treatment time. The cells cultured onto the meshes with plasma treatment for 6.2 s

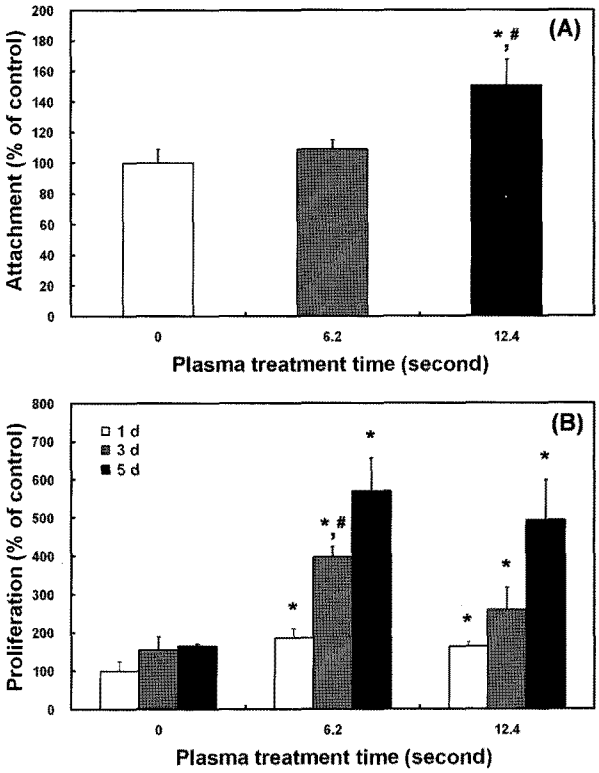


Figure 4. Effects of microwave-induced argon plasma treatment on cellular behaviors of nHAC-kn onto NSF meshes according to the treatment time. (A) Attachment of nHAC-kn onto non-treated and plasma-treated NSF meshes (*$p < 0.05$ vs. the non-treated and $^{\#}p < 0.05$ vs. the plasma-treated for 6.2 s, analyzed by a Student's *t*-test, n = 6). (B) Proliferation of nHAC-kn onto non-treated and plasma-treated NSF meshes (*$p < 0.05$ vs. the non-treated and $^{\#}p < 0.05$ vs. the plasma-treated for 12.4 s at the same time, analyzed by a Student's *t*-test, n = 6).

showed significantly ($p < 0.05$) better proliferation after 3 d of incubation than those with plasma treatment for 12.4 s. Nevertheless, the cell proliferation onto the plasma-treated meshes was significantly ($p < 0.05$) greater than that onto the non-treated meshes at each time point. This result suggests that the hydrophilic surface of the plasma-treated NSF meshes was more favorable for the cell spreading and growth than the hydrophobic surface of the non-treated meshes.

These results were then confirmed by SEM micrographs showing the cellular morphology of nHAC-kn cultured onto for 5 d NSF meshes treated without or with plasma (Figure 5). The cells on the non-treated mesh party covered the surface and spread with local attachment, as shown in Figures 5(A) and 5(B). In contrast, the cells on the plasma-treated meshes were well-grown and formed an almost single layer with maintaining the natural original shape and morphology of articular chondrocytes (Figures 5(C), 5(D)). It seemed that the increased surface roughness by plasma treatment might give a positive effect on cell attachment and growth. These

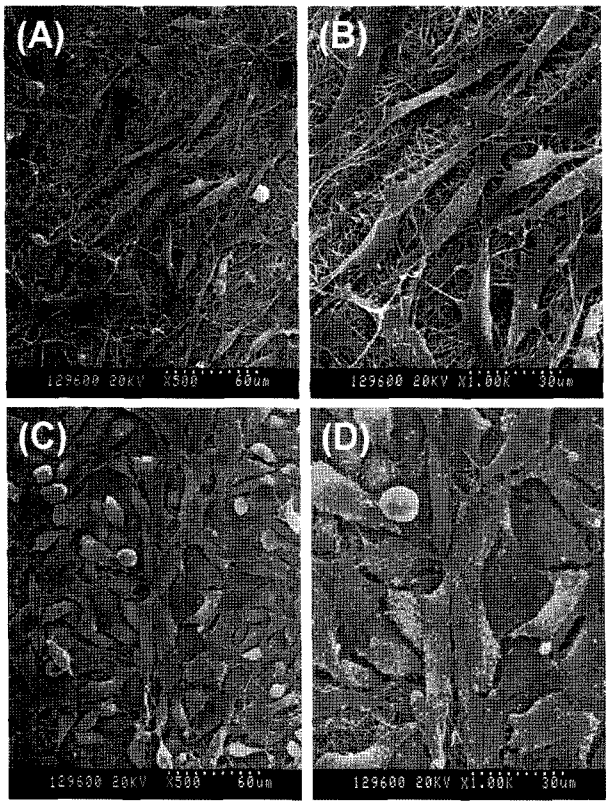


Figure 5. SEM micrographs of nHAC-kn cultured for 5 d onto NSF meshes treated without (A and B) or with (C and D) microwave-induced argon plasma for 6.2 s.

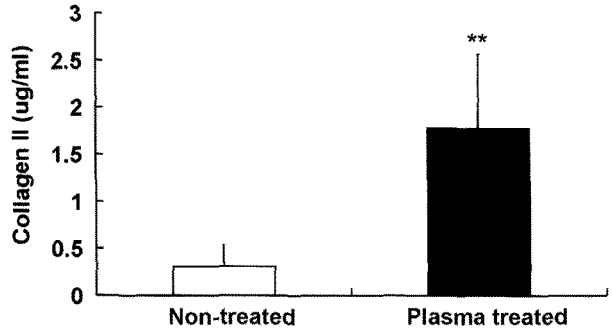


Figure 6. Native type II collagen of nHAC-kn cultured for 5 d onto NSF meshes treated without or with microwave-induced argon plasma for 6.2 s (**$p < 0.1$ vs. the non-treated, analyzed by a Student's t-test, n = 3).

results suggest that nHAC-kn are essentially compatible with the plasma-treated NSF meshes although the non-treated NSF is favorable to the cells as well. Recent report has shown that SF hydrogel-derived sponge was combined with freshly isolated rabbit chondrocytes for *in vitro* cartilage tissue engineering.[17] Furthermore, SF scaffolds has been shown to be used for *in vitro* cartilage tissue engineering in combination with mesenchymal stem cells or adult human chondrocytes.[18,19] Articular chondrocytes progressively undergo dedifferentiation into a spindle-shaped mesenchymal cellular phenotype in monolayers.[20] We examined the role of the plasma-treated NSF meshes in type II collagen expression, a marker of dedifferentiated chondrocytes. Type II Collagen Detection Kit (Chondrex, Redmound, WA) showed that type II collagen expression of the plasma-treated NSF meshes was higher than on the non-treated NSF meshes (Figure 6).

Conclusions

In conclusion, the results indicate that argon-plasma treatment is an appropriate technique to modify the surface properties of SF meshes to promote the attachment and proliferation of nHAC-kn. It is suggested that this approach can be helpful to apply to cartilage tissue engineering.

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