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Original Article

Biocompatibility between phbhhx films modified by phap-rgd fusion protein and human nasal septum chondrocytes

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Abstract: Objective: We discussed the biocompatibility between PHBHHx films modified by PhaP-RGD fusion protein and human nasal septum chondrocytes. Method: Human nasal septum chondrocytes cultured in vitro were inoculated to PHBHHx films modified by PhaP-RGD fusion protein for cultivation for 3 d and 7 d, respectively. Biocompatibility between PHBHHx films modified by PhaP-RGD fusion protein and human nasal septum chondrocytes was characterized by MTT colorimetry assay, DAPI staining, scanning electron microscopy and toluidine blue staining. Results: The water contact angle of PHBHHx films modified by PhaP-RGD fusion protein decreased and the hydrophilicity increased. Human nasal septum chondrocytes maintained a high proliferation rate on the films modified by PhaP-RGD fusion protein. After in vitro culture for 3 d, the chondrocytes growing on the modified films had a significantly higher viability than those on unmodified films. Toluidine blue staining revealed that PHBHHx films modified by PhaP-RGD fusion protein promoted the secretion of extracellular matrix glycosaminoglycans by chondrocytes. Conclusion: PHBHHx films modified by PhaP-RGD fusion protein had a better biocompatibility with chondrocytes. Therefore, modification by PhaP-RGD fusion protein represented a good surface biomodification method.

Keywords: PhaP-RGD fusion protein, PHBHHx, human nasal septum chondrocytes, tissue engineering

Introduction

Chondrocytes have limited self-repair ability and the repair of cartilage damage to the ears, nose, pharynx and larynx due to inflammation or congenital malformation is a great challenge. At present, defective cartilage of head and neck can be reconstructed by tissue engineering technique. Human nasal septum chondrocytes as hyaline cartilage is easily accessible and maintain good properties within 3 generations. These features make human nasal septum chondrocytes suitable to serve as seeder cells in cartilage repair by tissue engineering. Third generation PHA, namely, PHBHHx, is a natural biomaterial found in microorganisms and produced by microbial fermentation under disequilibrium conditions. Besides good biocompatibility and biodegradability, PHBHHx also possesses certain mechanical strength and thermoplasticity [1]. Localized on the surface of PHA, PhaP is a granule-binding protein with small molecular weight and amphiprotic property. RGD peptide can promote adhesion between cells and biomaterials [2, 3]. Relevant researches showed that PhaP-RGD fusion protein purified by protein engineering techniques improved the hydrophilicity of the surface of biomaterials and the adhesion of cells to biomaterials. Moreover, the differentiation of human mesenchymal stem cells into chondrocytes was enhanced by PhaP-RGD fusion protein [4].

We observed the in vitro growth of human nasal septum chondrocytes on the PHBHHx films modified by PhaP-RGD fusion protein. The effects of modification by PhaP-RGD fusion protein on hydrophilicity of PHBHHx films and the biocompatibility between PHBHHx films and chondrocytes were analyzed.

Materials and methods

Materials

Human nasal septum chondrocytes were harvested from the cartilage removed in nasal endoscopy. PHBHHx was provided by Prof. Chen Guo from Tsinghua University, and PHBHHx film was prepared by our research team. Expression of PhaP-RGD fusion protein was induced and protein purification was performed by our research team.

Methods

Isolation and in vitro culture of human nasal septum chondrocytes

Cartilage removed in nasal endoscopy (approved by hospital ethnic committee) was used. After the stripping of fascia covering the cartilage, three-step digestion method was performed. The digestion product was filtered and centrifuged with the supernatant discarded. The cells were resuspended in DMEM (H) containing 10% fetal bovine serum and then cultured at 37°C in a humidified 5% CO_2 incubator. Culture medium was replaced once after 48 h then at the frequency of once every 2 d. Cells growing 90% confluence after 1 w were digested by 0.5% trypsin and passaged at a 1:2 ratio.

Expression and purification of PhaP-RGD fusion protein

Recombinant *E. coli* BL21 (DE3) was selected for the expression of PhaP-RGD fusion protein. 100 ul of *E. coli* BL21 (DE3) carrying recombinant plasmid with PhaP-RGD gene was inoculated to 10 ml LB medium (5 mg/ml ampicillinum). The cells were cultured on a shaker at 37°C for 12 h and added with 100 ul 24 mg/ml ITPG for further culture for 5 h to induce the expression of fusion protein. After verification, Ni-NTA His Bind Purification Kit (Takara) was used to purify the target protein. SDS-PAGE and Western Blot were performed to identify the protein. *E. coli* BL21 (DE3) carrying no plasmid was taken as control (without the addition of ampicillin as inducer).

Preparation of PHBHHx films

PHBHHx films were prepared by solvent evaporation method according to the following procedures: 5 g of PHBHHx was weighed and dissolved in 100 ml of chloroform. The solution was heated in water bath at 60°C for 1 h after being sealed with plastic wrap. Fully dissolved biomaterial was evenly coated onto 10 glass culture plates with diameter of 9 cm, and the culture plates were covered by plastic wrap to prevent dust. Holes were made with needles to allow natural evaporation. The films were ready after 24-48 h.

Modification by PhaP-RGD fusion protein

PHBHHx films were soaked in 3.5 mg/ml purified PhaP-RGD solution and incubated at 4°C overnight. The proteins not bound to the films were washed off with PBS buffer.

Variation of water contact angle of PHBHHx films: Water contact angle was measured based on grouping as below: for group A (control group), no treatment was administered; for group B (PhaP-RGD fusion protein treatment group), the fusion protein was incubated at 4°C overnight. The PHBHHx films were cut into small pieces and treated by different methods. The treated films were washed with pure water, and the water was removed with filter paper before measurement. For both groups, the films were stuck to the specimen holder of the contact angle meter using double-sided adhesive tape. Onto the film 10 ul of pure water was added dropwise, and images were taken for the selected positions. The water contact angle was calculated according to the measurements.

Inoculation of human nasal septum chondrocytes to PHBHHx films

Before cell culture, unmodified PHBHHx films were soaked in 75% ethanol overnight. The films were irradiated by ultraviolet light on both sides, 1 h for each side, and then washed with PBS buffer three times. The films modified by PhaP-RGD fusion protein were washed with PBS buffer three times before culture cell. Using a 48-well plate, the chondrocytes were inoculated to the films before and after modification at the density of $2\times107/cm^2$. Cell growth directly on the culture plate was taken as control, with 3 replicates. The cells were cultured at 37°C in a humidified 5% CO_2 incubator for 3 d and 7 d, respectively.

Detection of cell proliferation and viability

After in vitro culture for 3 d, the cells were fixed in 4% paraformaldehyde for 30 min and washed

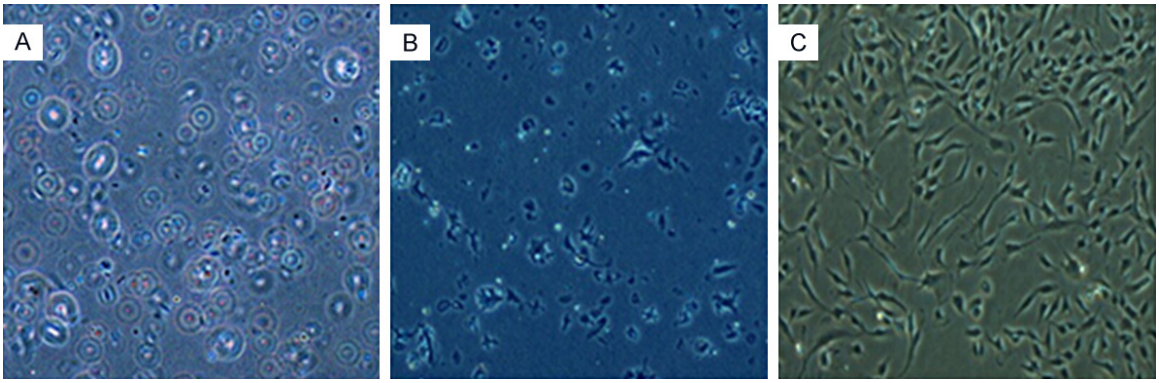

Figure 1. Morphology of human nasal septum chondrocytes (×40). A. Primary cells after in vitro culture for 2 h; B. Primary cells after in vitro culture for 48 h; C. Primary cells after in vitro culture for 7 d.

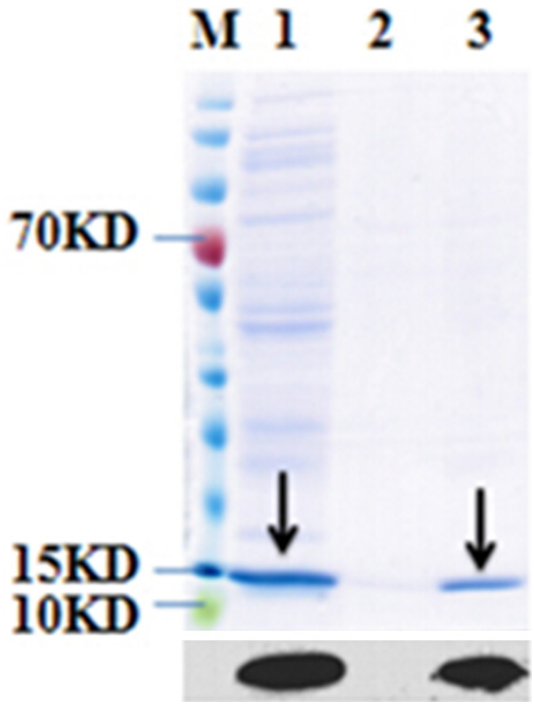

Figure 2. Identification and purification of PhaP-RGD fusion protein by SDS-PAGE and Western Blot. M: Marker; 1. Crude PhaP-RGD protein; 2. Liquid after washing Ni-NTA column bound with crude PhaP-RGD protein; 3. Protein after purification using Ni-NTA column.

with PBS buffer three times. Following DAPI staining for 5 min, washing was done with PBS buffer three times. Onto clean glass slide 2 ul of sterilized glycerol was added dropwise. PHBHHx and PHBV films were fixed on the glass slides and observed for chondrocyte proliferation.

Cell plates were cultured in vitro for 3 d and 7 d, respectively and 200 ul MTT solution (0.5 mg/ml) was added into each well. After further culture for 2-4 h, the culture medium was discarded, and the PHBHHx membrane was transferred to a new 48-well plate, with the side grown with cells facing upwards. For each well 200 ul DMSO was added, and the cells were oscillated on a shaker for 10 min. The liquid was added into labeled 96-well plate, 150 ul for each well. Absorbance value (A) was detected with a microplate reader at 492 nm.

Toludine blue staining

Cells were cultured in vitro for 7 d and washed with PBS buffer twice. Then they were fixed in 4% paraformaldehyde for 1 h or longer using a 6-well plate (4°C). Washing was done with tap water for 5 min and distilled water for 5 min successively. Staining was performed with 1% toludine blue for 2 h. Excess staining liquid was removed and the films were observed under the microscope.

Scanning electron microscopy

The cells growing on the films were observed under the scanning electron microscope after metal spraying.

Statistical analysis

Statistical analysis was performed using SPSS13.0, and $P<0.05$ was considered as statistically significant.

Results

Morphology and growth features of human nasal septum chondrocytes

Under the phase-difference microscope: Primary chondrocytes were round with a high

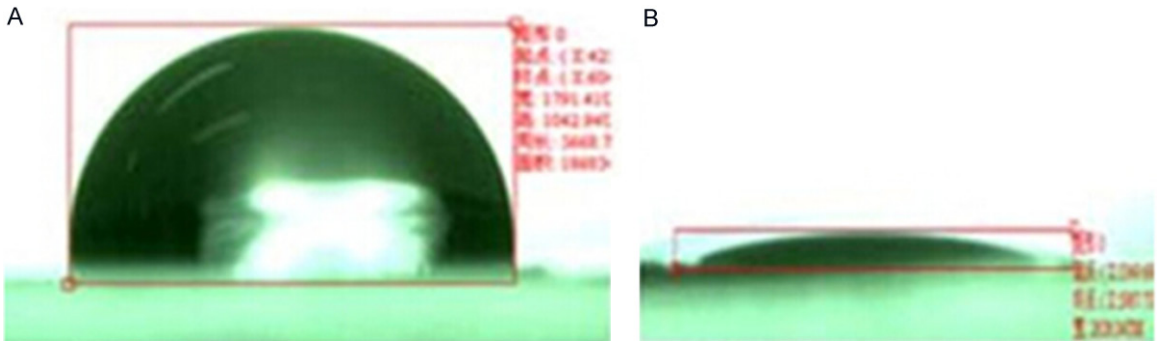

Figure 3. Measurement of water contact angle of PHBHHx films. A. Measurement of water contact angle of unmodified PHBHHx films; B. Measurement of water contact angle of PHBHHx modified by PhaP-RGD fusion protein.

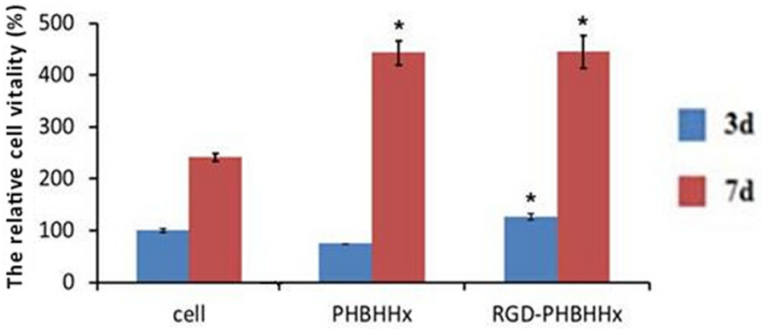

Figure 4. Cell viability on PHA films by MTT assay.

refractivity (**Figure 1A**). The cells adhered to wall after 2 days (**Figure 1B**) and began to extend since day 3. Initially the cells were spindle-shaped and later took the shape of irregular triangle or polygon. The cytoplasm contained round, dense granules (**Figure 1C**).

Expression and purification of PhaP-RGD fusion protein

After the verification of target protein, the proteins were purified by Ni-NTA column based on the fact that the His-tagged fusion protein can binds specifically to Ni. SDS-PAGE and Western Blot were performed for verification, with the results shown in **Figure 2**. It can be seen that a single fusion protein band was obtained after Ni-NTA column purification.

Measurement of water contact angle

Hydrophilicity of the modified films was measured by water contact angle. The results are shown in **Figure 3**. For control group and modification group, the water contact angle of PHBHHx films was 98.69° and 10.63°, respectively. The water contact angle decreased and the hydrophilicity of the films significantly after modification by protein fusion.

Proliferation and viability of chondrocytes on PHBHHx films modified by fusion protein

Cell viability on the modified PHBHHx films was detected by MTT assay. After culture for 3 d, the cell viability of modified PHBHHx films was higher than that of the other two groups. At 7 d, the cell viability of both modified and unmodified PHBHHx fimls was significantly higher than that of cells grown on culture plate. The cell viability on modified PHBHHx films was slightly higher than that on unmodified PHBHHx films. The above results indicated that PHBHHx films had no cytotoxicity on chondrocytes and that modification by PhaP-RGD fusion protein enhanced the cell viability on PHBHHx films (**Figure 4**).

DPAI staining was performed to determine cell proliferation. Compared with cell growth on culture plate and unmodified PHBHHx films, more cells were grown on the modified PHBHHx films (**Figure 5**).

Morphology of chondrocytes on unmodified PHBHHx films

Cell morphology was observed under the scanning electron microscope. It was found that all cells had normal morphology (**Figure 6A**, **6C**) with connections between cells and showed signs of fusion. Some cells began to grow into the pores on the surface of films or even spanned over the pores (**Figure 6B**, **6D**).

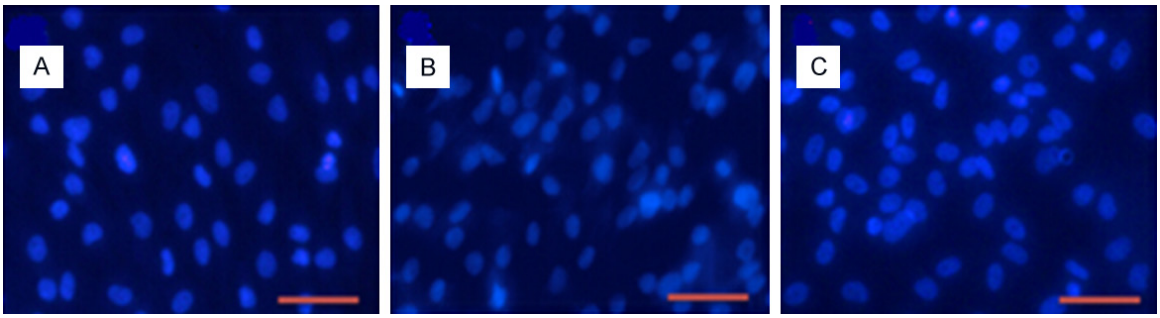

Figure 5. Adhesion of chondrocytes to films before and after modification by PhaP-RGD fusion protein. A. Chondrocytes grown on culture plate; B. Adhesion of chondrocytes to unmodified PHBHHx films; C. Adhesion of chondrocytes to modified PHBHHx films. The blue fluorescence denotes the nuclei labeled by DAPI (scale =100 μm).

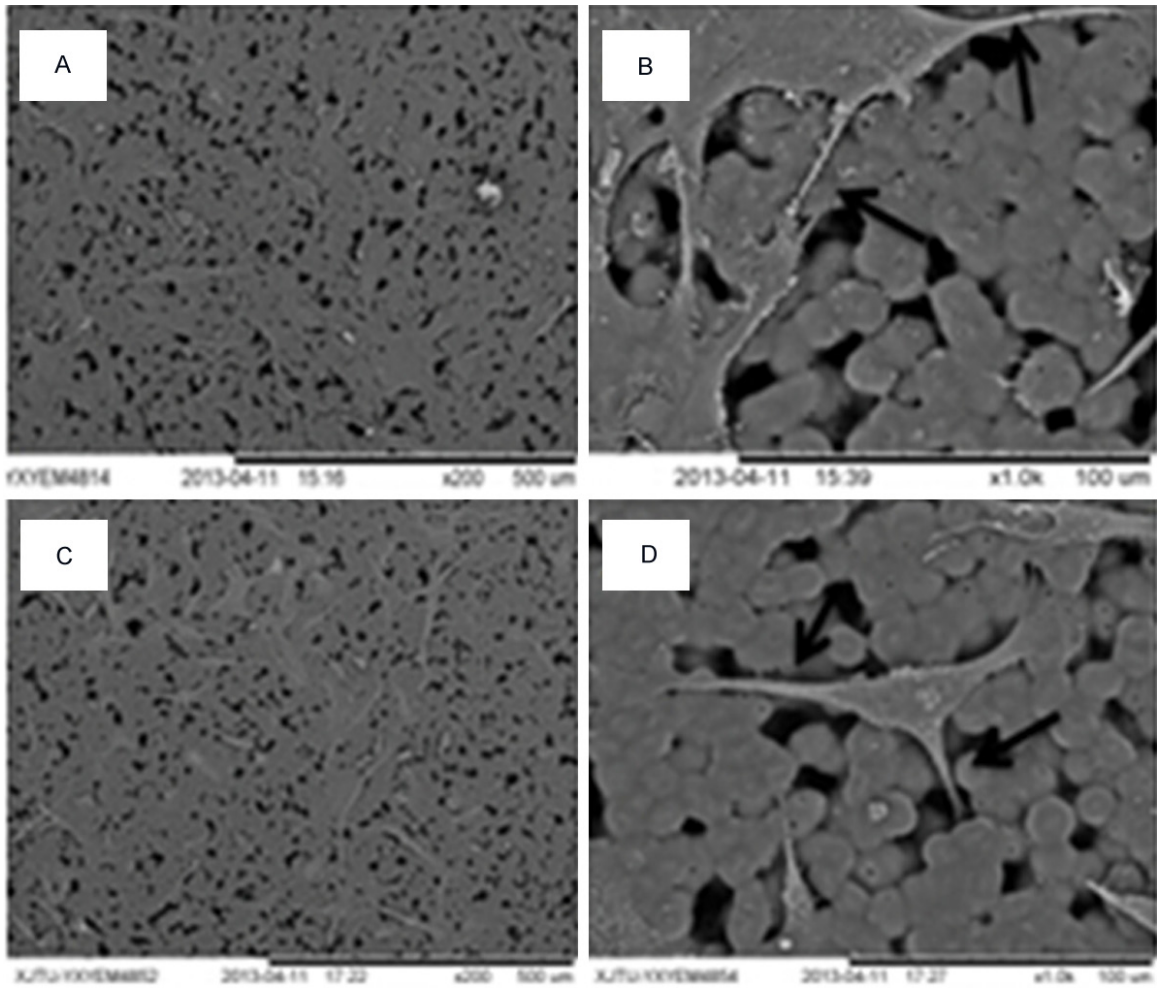

Figure 6. Morphology of chondrocytes on modified and unmodified PHBHHx films under scanning electron microscope. A-D. Cells on unmodified PHBHHx films after culture for 7 d. A, B. Morphology of chondrocytes on unmodified PHBHHx films (×200 and×1000). C, D. Morphology of chondrocytes on modified PHBHHx films (×200 and ×1000).

Extracellular matrix secretion

As shown by toludine blue staining, no metachroming was observed on the films not inoculated with cells (**Figure 7A**). Blue color given by metachroming was found on both modified and unmodified PHBHHx films. The cells were stained with a deeper color on modified PHBHHx films than on unmodified PHBHHx films (**Figure 7B**, **7C**). This indicated that modifi-

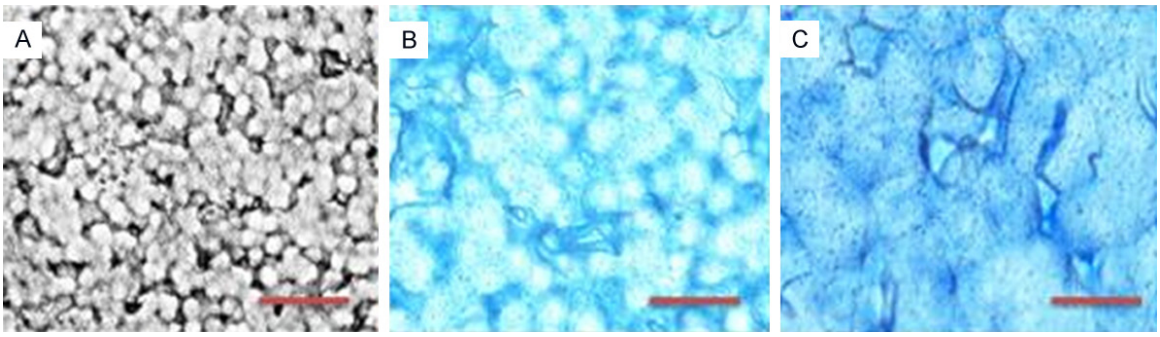

Figure 7. Secretion of extracellular matrix by chondrocytes on PHBHHx films. A. PHBHHx films (×10); B. Chondrocytes+PHBHHx films (×10); C. Chondrocytes+PhaP-RGD fusion protein+PHBHHx films (×20).

cation by PhaP-RGD fusion protein better promoted the secretion of extracellular matrix glycosaminoglycans.

Discussion

Cartilage of head and neck has limited self-repair ability and cartilage defects can be hardly corrected by surgical treatment. So far the repair of cartilage defects in the field of otolaryngology is still one of the greatest difficulties. Cartilage tissue engineering provides a good solution to this problem by first inoculating the seeder cells to the biomaterials to form a complex for subsequent transplantation. Ideal scaffolds for cartilage transplantation should be biodegradable, have certain mechanical strength and no cytotoxicity. More importantly, the scaffolds should promote the survival and proliferation of chondrocytes and help chondrocytes to maintain a normal phenotype without affecting cell viability [5-10]. PHBHHx has been extensively studied as scaffold material for tissue engineering due to its good biocompatibility. However, the strong hydrophobicity of PHBHHx as a polymeric biomaterial is a major defect, which makes surface modification necessary [11-14]. Ultraviolet light, plasma and ozone are usually used for surface modification of PHA through the mechanism of forming desired functional groups on material surface, thus changing the surface topology of the materials [15-17]. These methods impair the performance of biomaterials. Along with the progress in tissue engineering, specific recognition and modification have attracted an increasing attention. Cellular recognition of biomaterial surfaces plays an important role in tissue engineering as it is involved in cell adhesion to biomaterials as well as in cell proliferation and differentiation. The introduction of molecules or functional groups to biomaterial surfaces that can be specifically recognized by cells is an important method in terms of surface modification [18, 19]. RGD peptide can promote the adhesion of cells to biomaterials. In one study [20], PHBV scaffold coated with RGD improved cellular recognition and biocompatibility without affecting the biotin-binding performance. Through the fusion of RGD peptide and granule binding protein PhaP on PHA surface, the biocompatibility of scaffolds was improved, and cell differentiation was promoted with the maintenance of a normal morphology [21]. The fusion of PhaP protein and RGD peptide proves to be an effective method of surface modification that enhances biocompatibility of the biomaterials.

We introduced PhaP-RGD fusion protein to the surface of PHBHHx films. According to measurements, the water contact angle of PHBHHx films modified by PhaP-RGD fusion protein was obviously reduced. This indicated that PhaP-RGD fusion protein improved the hydrophobicity of PHBHHx films. Human nasal septum chondrocytes were seeded to PHBHHx films and cultured in vitro for 3 d. The adherence of cells to PHBHHx films proved good cellular affinity of PHBHHx films. Cell adhesion to PHBHHx films coated by PhaP-RGD fusion protein was made easier by the reduction in hydrophobicity. A large number of cells proliferated on both modified and unmodified films 7 days later. Moreover, a large quantity of extracellular matrix was seen around cells on modified films, indicating a good maintenance of the properties of chondrocytes. As demonstrated by in vitro experiments, modification by PhaP-RGD fusion protein promoted the biocompatibility between

PHBHHx films and human nasal septum chondrocytes.

Our experiment indicated that PHBHHx films modified by PhaP-RGD fusion protein as scaffold for the growth of human nasal septum chondrocytes can help chondrocytes maintain normal phenotype and promote cell viability. The findings provide convincing evidence of the suitability of modified PHBHHx films used as scaffold for chondrocyte transplantation.

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Disclosure of conflict of interest

None.

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