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Coating of hydrophobins on three-dimensional electrospun poly(lactic-co-glycolic acid) scaffolds for cell adhesion

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Abstract
Surface modification with hydrophobins is very important for cell adhesion in its applications in biosensor fabrication. In this study, we modified the surface of three-dimensional electrospun poly(lactide-co-glycolide) (PLGA) scaffolds with hydrophobin HFBI and collagen, and investigated its applications for cell adhesion. We found that HFBI could not only improve the hydrophilicity of the three-dimensional electrospun PLGA scaffolds but also endow the electrospun PLGA scaffolds with water permeability. This permeability should be attributed to both the hydrophilicity of the modified PLGA surface and the large positive capillary effect induced by the microstructures. Further experiment indicated that HFBI modification could improve collagen immobilization on the electrospun PLGA scaffolds and the HFBI/collagen modified electrospun PLGA scaffolds showed higher efficiency in promoting cell adhesion than the native PLGA scaffolds. This finding should be of potential application in biosensor device fabrication.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Wettability of solid surfaces is very important to their applications, which is governed by both chemical composition and topographic structure [1–4]. Surface wettability conversion can be achieved with amphiphilic hydrophobins, e.g. it has been well documented that hydrophobins can improve the hydrophilicity of the poly(dimethylsiloxane) (PDMS) surface [5, 6] and the silicon surface [7]. The modified surfaces are reported to be more compatible for biomaterial immobilization, such as protein adsorption [5] and cell adhesion [8]. However, most researches of surface modification using hydrophobins are mainly focused on the static wettability conversion of two-dimensional surfaces; while the researches about hydrophobin modification on three-dimensional PLGA scaffolds have rarely been reported.

As far as a three-dimensional structure is concerned, the PLGA scaffolds made by electrospinning are a typical substrate. Electrospinning is a technique which applies an electrical force on a polymer solution to produce fibers of polymers. When the electrical force is lager than the surface tension force of the polymer solution, the fibers of polymers are extruded from the solution and weaved into membranes. This technique can produce fibers with diameters ranging from 50 nm to 500 nm. These fibers interconnect with each other to form porous scaffolds, which are ideal substrates for drug delivery and cell culture applications [9, 10]. A commonly used polymer in the electrospinning technique is

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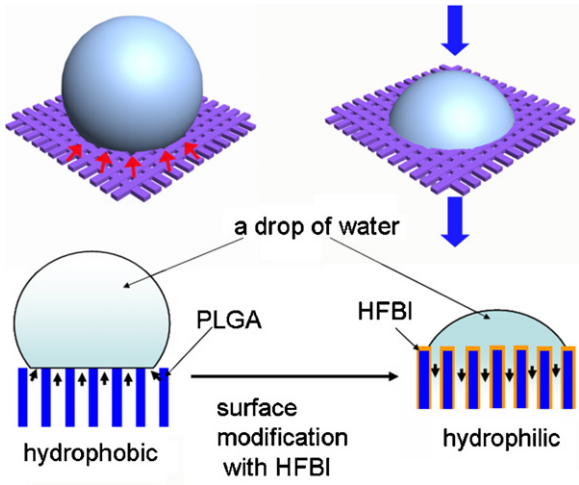
PLGA, which has been used as a biodegradable material in tissue engineering [11, 12] and gene therapeutic fields [10]. It has been reported that the three-dimensional electrospun PLGA scaffold is superior to the two-dimensional PLGA film in tissue engineering due to its unique morphology, e.g. the electrospun PLGA scaffolds are more suitable for cell colonization and bone tissue formation since the morphology of the three-dimensional PLGA scaffolds is similar to the trabecular bone [13]. However, the three-dimensional structure of PLGA scaffolds also increases their hydrophobicity, which will cause some trouble in their application. For instance, the hydrophobicity will cause a poor distribution of protein solutions on the PLGA surface and thus weaken the immobilization of the proteins. In addition, the electrospun PLGA scaffolds are not water permeable due to the negative capillary effect, which is caused by the hydrophobicity of the PLGA surface. When PLGA scaffolds are placed into the cell culture medium, the cell culture medium cannot enter the PLGA scaffolds easily and air bubbles will be trapped in the PLGA scaffolds. It is urgent to find a method to increase the hydrophilicity and the water permeability of the PLGA scaffolds.

Hydrophobins are a family of small, cysteine-rich and amphipathic fungal proteins [14], which can self-assemble into amphiphilic membranes at hydrophilic–hydrophobic interfaces to convert the surface from hydrophilic to hydrophobic and vice versa [15]. Hydrophobins have unique self-assembling behavior due to their remarkable structural characteristics [15]. Their molecular weight is about 7–15 kDa and their amino acid sequence has hydrophilic and hydrophobic regions. Hydrophobins can be classified into two classes according to their self-assembly patterns and solubility characteristics [16]. Class I hydrophobins, such as SC3, can form random patterns of nanoscopic rodlets and bundles when dried on the hydrophilic surfaces [17, 18]. Class II hydrophobins, such as HFBI and HFBII, can form highly ordered monolayer films and crystalline fibrils when assembling at an interface [19, 20]. In addition, the stabilities of their self-assemblies are different. Class I hydrophobins are very insoluble, such as SC3, which can only be dissolved in strong acids such as formic acid or trifluoroacetic acid (TFA) [21], while class II hydrophobins are easier to be dissolved and can be dissolved in ethanol or sodium dodecyl sulfate [22].In this study, the electrospun PLGA scaffolds were modified with HFBI, and their wettability and water permeability were monitored by measuring the water contact angel (WCA). Data showed that HFBI could not only increase the hydrophilicity of the electrospun PLGA scaffolds, but also endow the PLGA scaffolds with water permeability. Water permeability is believed to be the macroscopic behavior caused by the large positive capillary effect, which is induced by both the hydrophilicity and the micro- or nano-structure of the modified PLGA scaffolds (scheme 1). Due to the improved hydrophilicity and water permeability, the modified electrospun PLGA scaffolds were more suitable for collagen immobilization. Further experiment showed that the HFBI/collagen modified PLGA scaffolds could promote cell adhesion much better than the native PLGA scaffolds. Herein,



Scheme 1. Schematic illustration showing the wettability and water permeability conversion on three-dimensional electrospun PLGA scaffolds by HFBI. Due to the large negative capillary effect, the native electrospun PLGA scaffolds are not water permeable. Self-assembly of HFBI converts the PLGA surface from hydrophobic to hydrophilic. Due to the positive capillary effect, the three-dimensional PLGA scaffolds are endowed with water permeability.

HFBI modification can be used as a method to improve the hydrophobicity and the water permeability of the electrospun PLGA scaffolds for protein immobilization and cell adhesion.

2. Materials and methods

2.1. General

Rat-tail type I collagen was purchased from Sigma Corp. Fetal bovine serum was purchased from Tianjin Shengda Corp. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen Corp. The three-dimensional electrospun PLGA scaffolds (LA/GA = 75:25) and the two-dimensional PLGA films (LA/GA = 75:25) were purchased from Jinan Mingtian Corp. Hoechest33342 was purchased from Dingguo Corp. Hydrophobin II HFBI was generously donated by Professor Min-Qiang Qiao in Collage of Life Science, Nankai University, and was diluted to 100 μg mL^{-1} with water before use.

2.2. Surface modification with HFBI and collagen

The electrospun PLGA scaffolds and the PLGA films were cut into 1 cm^2 squares. Both substrates were incubated with HFBI solutions for 20 min and dried with a stream of nitrogen. Then the substrates were carefully rinsed with distilled water several times to remove the unbound HFBI. The resulting substrates were dried under a stream of nitrogen. Collagen modification of the electrospun PLGA scaffolds and HFBI modification of the PLGA films were performed in the same way.

2.3. Water contact angle measurement

The wettability of the surface was investigated by water contact angle (WCA) measurement. 2 μL of water was dripped on

the surfaces under ambient conditions with an optical contact angle meter (HARKE-SPCA). The image was captured at 1 s, 4 s and 8 s after the water droplet was dripped on the surface. The WCA data were presented as the average value of triple individual measurements at different locations of the same substrate ± mean square deviation.

2.4. Scanning electron microscopy observation

The native PLGA scaffolds, the PLGA scaffolds modified with HFBI, the PLGA scaffolds modified with collagen and the PLGA scaffolds modified with collagen/HFBI were prepared in the same way as mentioned above. Scanning electron microscopy (SEM) observation was performed using a scanning electronic microscope (SHI MADZU SS-550) equipped with SS-550 Software Version 2.05.

2.5. Cell adhesion

293 T cells (human embryonic kidney cell line) were routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum. The cells were rinsed with phosphate buffered saline (PBS), treated with trypsin-EDTA (0.25%) and incubated for 10 min (37 °C) in a 5% CO_2 incubator to be detached. The cell suspension was supplemented with the same volume of DMEM with 10% (v/v) serum to stop the reaction of trypsin. The cell suspension was then centrifuged at 800 rpm to obtain the cells.

The cell adhesion study in the absence of serum was performed as follows. The cells were resuspended in serum-free DMEM after centrifuging. The PLGA scaffolds were modified by HFBI and then by collagen in the same way as described above. The modified PLGA scaffolds were placed in the wells of 24-well plate. The cell suspension was dripped into the wells and the cells were incubated in a 5% CO_2 incubator at 37 °C for 24 h. The native PLGA scaffolds were used as a control. The experiment was carefully controlled to make sure that each well has the same number of cells.

The cell adhesion study in the presence of serum was performed as follows. The cells were resuspended in DMEM with 10% (v/v) serum after centrifuging. The PLGA scaffolds were modified by HFBI and then by collagen in the same way as described above. The modified PLGA scaffolds were placed in the wells of 24-well plate. The cell suspension was dripped into the wells and the cells were incubated in a 5% CO_2 incubator at 37 °C for 24 h. The native PLGA scaffolds were used as a control. The experiment was carefully controlled to make sure that each well has the same number of cells.

After 24 h incubation, the cells were dyed with Hoechest33342 following its instructions. The cell number was counted under a fluorescent microscope TE 2000-U (Nikon Japan) equipped with Spot software. The results were presented as the average value of six individual measurements ± mean square deviation.

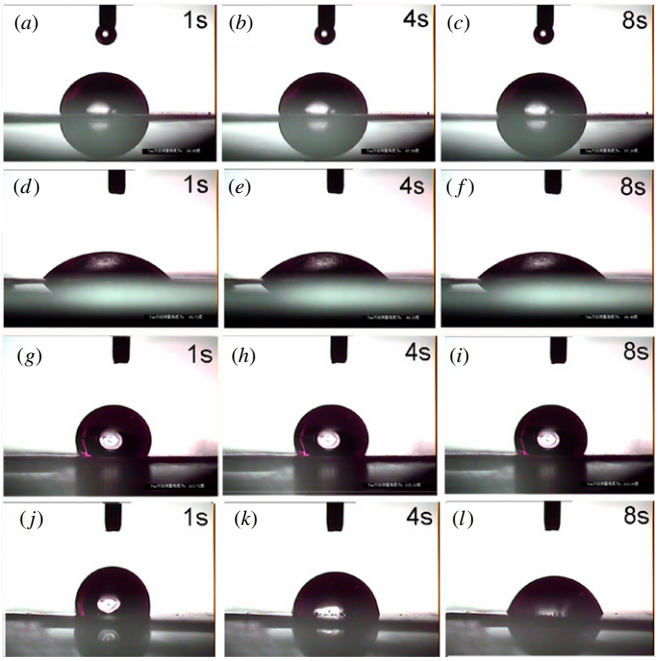


Figure 1. Consecutive frames of a water drop being dripped on a flat PGLA film surface (*a*)–(*f*) and electrospun PLGA scaffolds (*g*)–(*l*). (*a*)–(*c*) is the native PLGA film. (*d*)–(*f*) is PLGA film modified with HFBI. (*g*)–(*i*) is electrospun PLGA scaffolds. (*j*)–(*l*) is electrospun PLGA scaffolds modified with HFBI. The decrease of the water drop (*j*)–(*l*) means permeation of the water drop into the PLGA scaffolds.

3. Results and discussion

3.1. Surface wettability conversion by HFBI

Surface wettability conversion induced by hydrophobin HFBI on the three-dimensional PLGA scaffolds was investigated via measuring their WCAs. In order to investigate the influence of the three-dimensional structure on the hydrophobicity of the PLGA scaffold, the wettability conversion on a three-dimensional PLGA film induced by HFBI was also investigated as a comparison (figures 1 and 2).

The WCA of the native PLGA film was about 92° and the WCA of the electrospun PLGA scaffolds was about 110°. The enhanced hydrophobicity of the PLGA scaffolds was supposed to be caused by their three-dimensional structure rather than by their materials, since the composition of the electrospun PLGA scaffolds was the same as that of the PLGA film. Theoretical investigation indicates that the surfaces' micro-structure will have a significant influence on their macroscopic wettability. As shown in Wenzel's equation [23],

$$\cos\theta' = r\cos\theta \tag{1}$$

where θ' is the apparent WCA on a rough surface and θ is the intrinsic WCA on a flat surface, the surface roughness (r) can enhance both the hydrophilicity on a hydrophilic surface and the hydrophobicity on a hydrophobic surface. That means the micro- or nano-structure can make a hydrophobic surface more hydrophobic rather than more hydrophilic, and vice versa. Wenzel's equation is suitable to explain most phenomena in surface wettability conversion; however, it meets certain

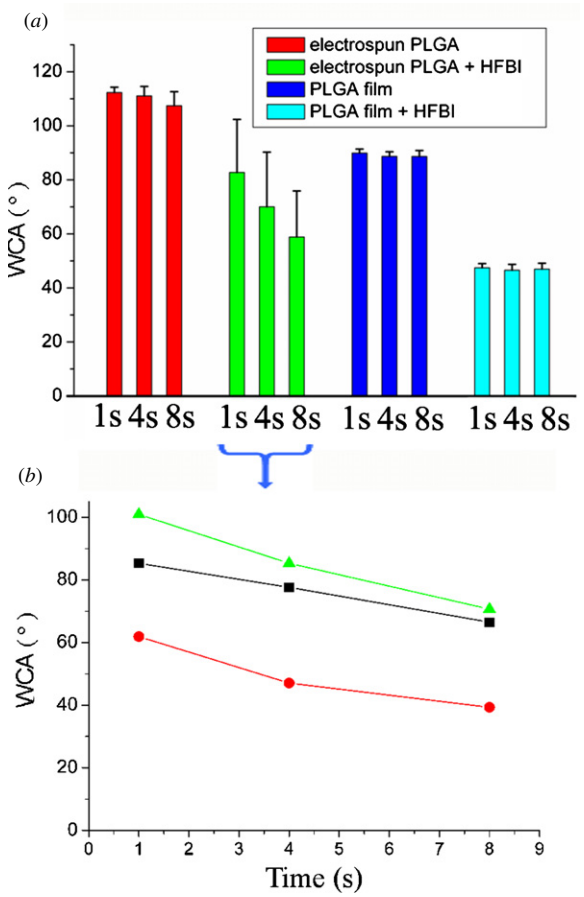


Figure 2. (*a*) WCAs measurement on the electrospun PLGA scaffold, the HFBI modified electrospun PLGA scaffold, the PLGA film and the HFBI modified PLGA film. We recorded the data at 1 s, 4 s and 8 s after the water was dripped on the surfaces. (*b*) Three WCA measurements on the HFBI modified electrospun PLGA scaffolds.

difficulties in some cases, in which air bubbles are trapped between the water surface and the solid surface due to the nano- or micro-structures. The theoretical research is enriched by the modified Cassle's equation [24],

$$\cos\theta' = f\cos\theta - (1 - f), \tag{2}$$

where f is the fraction of the solid/water interface. This equation means that the complex three-dimensional structure of the surface may trap some air bubbles on the interface between water and the material. Consequently the water drop can only contact with a small area of the micrometrescale surface, as a result of which the microscopic surface hydrophobicity is greatly enhanced. The three-dimensional structure of the electrospun PLGA scaffolds will trap air bubbles between the PLGA surface and the water. Consequently, the water droplet could only contact PLGA in the small areas between these air bubbles, which would greatly increase the hydrophobicity.

Water permeability is presented as the entrance of the water droplet into the substrate, which can also be presented as the changes of the WCAs. Consequently, we monitored the WCAs changes at 1 s, 4 s and 8 s after the water droplet was dripped onto the substrate. The WCAs of the native PLGA scaffolds did not vary obviously in an 8 s time period. That means the native electrospun PLGA scaffolds were not water permeable. The electrospun PLGA scaffolds are composed of PLGA fibers. The interspaces between these PLGA fibers form a lot of interconnected tunnels. Judging from the WCAs of the native PLGA film, the surface of the PLGA fibers should be hydrophobic. Then there will be a negative capillary effect, which is supposed to play an important role in prohibiting water entering the electrospun PLGA scaffolds.

After HFBI coating, the WCAs of both the electrospun PLGA scaffolds and the PLGA film decreased dramatically. The changes of WCAs were supposed to be the self-assembly of the HFBI on the PLGA surface. As reported, the HFBI is an amphiphilic protein, the protein sequence of which contains hydrophobic region and hydrophilic region [5]. Once HFBI self-assembles on a hydrophobic surface, the hydrophobic region will face the hydrophobic surface, leaving the hydrophilic region exposed to the outsides, which will subsequently change the surface from a hydrophobic one to a hydrophilic one.

It was noteworthy that the WCAs on the HFBI modified PLGA scaffolds decreased greatly in 8 s. At the same time the volume of the water droplet decreased quickly. That means the water entered the electrospun PLGA scaffolds. Surface modification with HFBI can effectively convert the PLGA film from hydrophobic to hydrophilic. It is reasonable to deduce that the surfaces of the HFBI modified PLGA fibers are also hydrophilic. In this case, there will be a positive capillary effect when water enters the interspaces of the PLGA fibers, which should contribute greatly to the water permeability of the HFBI modified PLGA scaffolds.

In figure 2(*b*), the WCAs of the HFBI modified PLGA scaffolds did not repeat well in three measurements. We supposed that the phenomenon was caused by both the texture of the electrospun PLGA scaffolds and the limitation of the measuring equipment. The textures of three PLGA scaffolds cannot be exactly the same, so their capillary effect should be more or less different. Consequently, their data will be different from each other. Moreover, the water droplet entered the PLGA scaffolds very rapidly. It is hard to control the time for image capturing very exactly, which will also cause some difference in three measurements. The rapid entrance of water droplet into the PLGA scaffolds indicated that the water permeability of the HFBI modified PLGA scaffolds was very good.

The excellent water permeability of the electrospun PLGA scaffolds should be meaningful for their applications. The native PLGA scaffolds are water repellent. When we use electrospun PLGA scaffolds as the substrate for cell adhesion, the cell culture medium can not enter the electrospun PLGA scaffolds easily. There are many air bubbles trapped in the microstructure of the PLGA scaffolds. HFBI modification can endow the PLGA scaffolds with good water permeability, which should be able to solve this problem.

3.2. Protein immobilization

Surface modification with HFBI endowed the electrospun PLGA scaffolds with not only water permeability but also

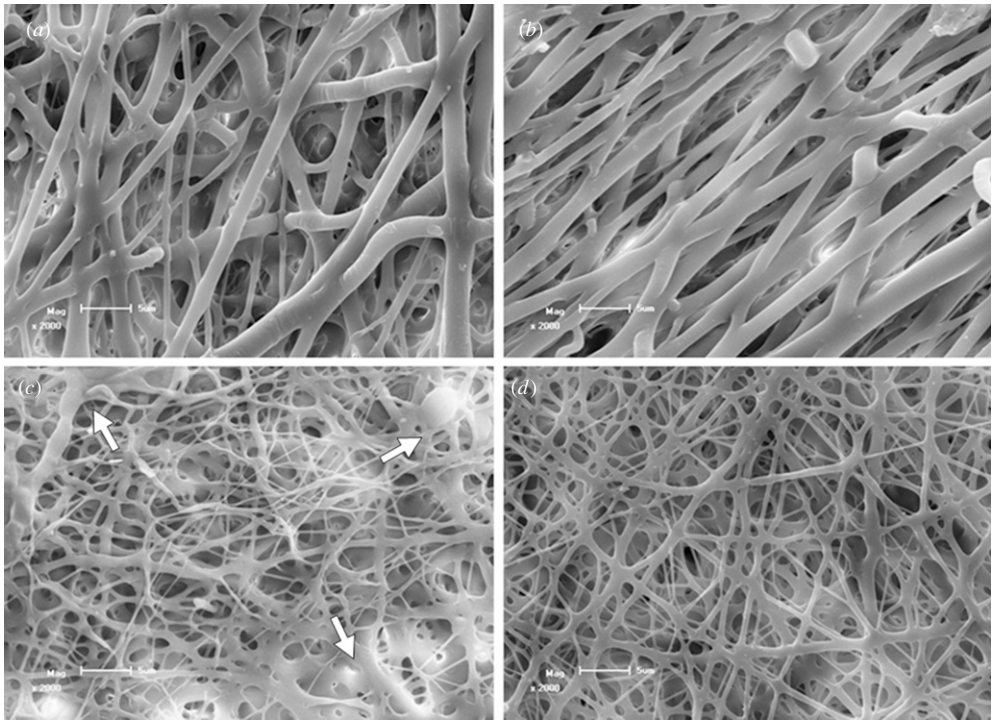


Figure 3. SEM images depicting HFBI modification on the electropun PLGA scaffolds. (*a*) The native PLGA scaffolds. (*b*) The HFBI modified PLGA scaffolds. (*c*) The collagen coated HFBI modified PLGA scaffolds. (*d*) The collagen directly coated PLGA scaffolds. The length of the scale bar in each image is 5 μm.

hydrophilicity, which should facilitate the protein adhesion on the electrospun PLGA scaffolds. In this study, we used collagen as an example to investigate influence of HFBI modification on protein immobilization on the PLGA scaffolds.

SEM images showed that the electrospun PLGA scaffolds were composed of many PLGA fibers, the width of which was about several hundred nanometers (figure 3(*a*)). Due to the limitation of the electrospinning technique, the diameters of these PLGA fibers varied obviously. Especially the fibers in figure 3(*a*) which seemed several times thicker than those in figure 3(*c*). However, they are from the same PLGA scaffolds and of the same scale. In fact, there are some thinner fibers in figure 3(*a*), the diameter of which is similar to that in figure 3(*c*). The variance of the fibers' size did not influence our investigation about the surface modification on PLGA scaffolds.

After HFBI modification the morphology of the PLGA fibers did not changes discriminably (figure 3(*b*)). The HFBI was a small protein and the concentration of HFBI was not very high, so HFBI modification did not change the surface morphology obviously. Collagen solution was coated on the HFBI modified PLGA scaffolds and the native PLGA scaffold, respectively. The collagen formed thick layers on the surface of the PLGA scaffolds modified with HFBI (figure 3(*c*)). The layer not only existed on the surface of the PLGA scaffolds but also filled some interspaces between the PLGA fibers. In comparison, the collagen did not form an obvious structure on the native PLGA scaffolds (figure 3(*d*)). The result indicated that surface modification with HFBI on the three-dimensional electrospun PLGA scaffolds could facilitate the immobilization of collagen. The self-assembly of HFBI on the surface of PLGA could effectively increase the hydrophilicity of the electrospun PLGA scaffolds. As a result, the collagen solution could be well distributed on the PLGA scaffold surface. In addition, the HFBI modification endowed the PLGA scaffolds with good water permeability, which would facilitate the entrance of collagen solution into the PLGA scaffolds. Consequently, collagen could be better immobilized on the PLGA scaffolds after HFBI modification.

3.3. Cell adhesion

Electrospun PLGA scaffolds are usually used as the substrate for cell growth in tissue engineering field [11, 13]. HFBI modification on the three-dimensional PLGA scaffolds improved the immobilization of collagen on the PLGA scaffolds. Because collagen can promote cell adhesion, the improved collagen immobilization on the PLGA scaffolds should be useful for cell adhesion.

The superiority of HFBI/collagen modified electrospun PLGA scaffolds in promoting cell adhesion was investigated experimentally in this study. The 293T fibroblast cells were cultured on the HFBI/collagen coated PLGA scaffolds and the native HFBI/collagen coated PLGA scaffolds, respectively. After 24 h, the cell number on each substrate was calculated. In the previous report, the serum in the cell culture medium can support cell adhesion [25]. If the cell culture medium contains some serum, the function of collagen will be weakened. Consequently, we cultured the cells in DMEM with 10%

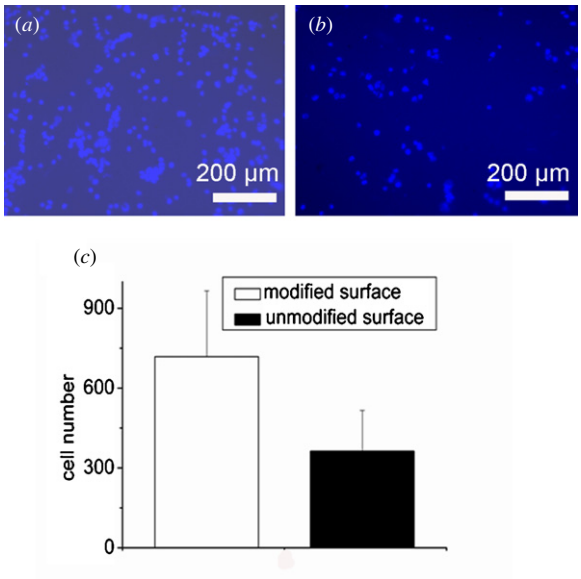


Figure 4. Cell adhesion on (*a*) the HFBI/collagen modified PLGA scaffolds and (*b*) the native PLGA scaffolds. (*c*) Statistical calculation of the cell number on the HFBI/collagen modified PLGA scaffolds and the native PLGA scaffolds. The cells were cultured in serum free DMEM.

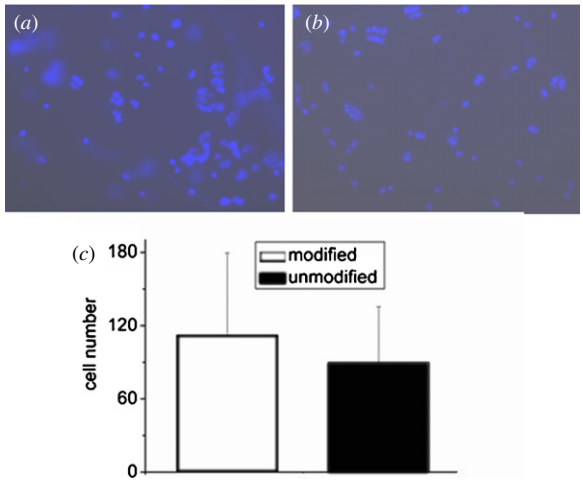


Figure 5. Cell viability on (*a*) the HFBI/collagen modified PLGA scaffolds and (*b*) the native PLGA scaffolds. (*c*) Statistical calculation of the cell number on the HFBI/collagen modified PLGA scaffolds and the native PLGA scaffolds. Cells were cultured in DMEM with 10% serum.

serum and serum-free DMEM, respectively, to investigate the influence of serum on the cell growth.

When the cells were cultured in serum-free DMEM, the influence of the serum was expelled and the cell growth was mainly influenced by HFBI/collagen modification. As shown in figure 4, there are more cells, which presented as the blue spots under a fluorescent microscope, on the HFBI/collagen modified PLGA scaffolds than on the native PLGA scaffolds. This result indicated that HFBI/collagen modified PLGA scaffolds were more suitable for cell adhesion. The improved cell adhesion of HFBI and collagen modified PLGA scaffolds should be partially attributed to the function of collagen in promoting cell adhesion [8]. In addition, HFBI modification endowed the PLGA scaffolds water permeability and would facilitate the cell culture medium entering the PLGA scaffolds. In this case, the cells could be better nourished by getting into contact with more culture medium from the interspaces in the PLGA scaffolds, which may also contribute to the enhanced cell adhesion on the modified PLGA scaffolds.

The result also indicates that collagen and HFBI can be well immobilized on the electrospun PLGA scaffolds. HFBI belongs to the class II hydrophobins. Previous research shows that the class II hydrophobins are easier to be dissolved than class I hydrophobins [22]. Consequently, the stability of HFBI self-assembly in cell culture medium is crucial for their application in cell culture. Although HFBI has been proved to be able to self-assemble on a glass surface [26], a PDMS surface [8] and a mica surface [5], whether HFBI mediated collagen immobilization on the three-dimensional PLGA scaffolds can be stable in cell culture medium was still unknown before this experiment. Since cells were cultured in a liquid environment at 37 °C for 24 h, it would be more difficult for the stable immobilization of HFBI and collagen. The metabolism of cells, the accelerated movement of water molecules at high temperature and the long time cell culture will inevitably do harm to the stability. This study experimentally proved the feasibility of immobilizing collagen on the PLGA scaffolds by HFBI modification and should contribute to further researches concerning cell culture on the PLGA scaffolds.

We also conducted a similar experiment, in which the serum was included in the cell culture medium. As shown in figure 5, the HFBI/collagen modified PLGA scaffolds were more suitable for supporting cell adhesion than the native PLGA scaffolds. But the difference between the cell numbers on the modified and the native PLGA scaffolds in this experiment was not as large as that in the serum-free experiment. Serum can promote cell adhesion. In this case, the function of collagen was made up by the serum on the native PLGA scaffolds. Consequently the difference was reduced.

4. Conclusions

HFBI modification of three-dimensional electrospun PLGA scaffolds and its applications in promoting protein immobilization and cell adhesion were investigated. HFBI could not only increase the hydrophilicity of the three-dimensional electrospun PLGA scaffolds, but also endow them with water permeability. Further experiment showed that the HFBI modification could promote collagen immobilization on the electrospun PLGA scaffolds. The HFBI/collagen modified PLGA scaffolds had better properties for cell adhesion. This study developed a new method to convert the wettability and water permeability of three-dimensional PLGA scaffolds by HFBI modification for protein immobilization and cell adhesion, which should be of potential value in the field of biosensor fabrication and tissue engineering.

Acknowledgments

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