Materials Research Express

OPEN ACCESS

PAPER

Structure and properties of PVA/silk fibroin hydrogels and their effects on growth behavior of various cell types

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Keywords: hydrogel, Poly(vinyl alcohol), silk fibroin, cell behavior regulation

Supplementary material for this article is available online

Abstract

Controllable regulation of cell behavior is one of the most important factors conducive to the restoration of tissue functions. Recently, various strategies have been developed using physical or chemical cues. Although these techniques are effective, the high cost and complex fabrication procedures impede their application. In this study, we used a low cost and simple strategy to fabricate PVA/silk fibroin composite hydrogels using a cyclic freeze-thaw method. With the increase of freeze-thaw cycles, the pore size of hydrogels decreased, the elastic modulus increased, and the swelling rate decreased. Furthermore, we chose two shapes of model cells, a spindle using bone marrow mesenchymal stem cells and smooth muscle cells, and a round shape using BV2 microglial cells. PVA/silk fibroin composite hydrogels inhibited the adhesion and proliferation of stem cells and muscle cells and changed their cell shape from spindle to round, maintained the initial round shape of BV2 microglial cells, and promoted the proliferation of BV2 microglial cells. These results demonstrate that PVA/silk fibroin composite hydrogels can be used as a novel hydrogel system to regulate cell behavior.

1. Introduction

Interactions between cells and materials are one of the most important topics in biomedical engineering. Among these interactions, effective regulation of cell behavior is very important, which is related to the fate of cells and even the repair and reconstruction of tissues. Recently, various techniques have been developed to regulate cell behavior. These strategies involve stiffness and viscoelasticity [1], stress relaxation [2, 3], micropatterning [4, 5], degradation rates [6, 7], chemical groups [8], and the length of polymer brushes [9]. However, the introduction of toxic residues, such as monomers, initiators, precursors, and crosslinkers, is still inevitable. In addition, the high cost and complex fabrication procedures restrict or delay the clinical application of scaffolds. Thus, a cost-effective and facile effective method is urgently needed to regulate cell behavior.

Polyvinyl alcohol (PVA) hydrogels possess excellent biocompatibility, high strength, water retention, and a porous structure [10–12]. However, the lack of biological activity does not support cell adhesion [13]. The intrinsic property of non-adhesion of cells to PVA hydrogels can be improved by introducing natural polymers such as silk fibroin. Silk fibroin contains an amino acid sequence that facilitates cell growth and migration [14]. In the present study, PVA/silk fibroin composite hydrogels were fabricated using a simple cyclic freeze-thaw method. The structure and properties of hydrogels, such as pore size, elastic modulus, and swelling ratio, were
evaluated systematically. Furthermore, we chose model cells with two different shapes, spindle and round, namely bone marrow mesenchymal stem cells and smooth muscle cells as the spindle model cell and BV2 microglial cells as immune cells with a round shape. Interestingly, cells with different shapes were selective growth on the surface of PVA/silk fibroin composite hydrogels (scheme 1).

2. Materials and methods

2.1. Materials
PVA (n = 1750 ± 50) was obtained from Sinopharm Chemical Reagent Co., Ltd (China). Silkworm cocoons were obtained from Tongxiang Yuantong Silk Co., Ltd (China). Dialysis bags were acquired from Solarbio Life Sciences Co., Ltd (China). All other agents or solvents with analytical reagent grade were purchased from Aladdin Co., Ltd (China).

2.2. Preparation of the silk fibroin solution
Silkworm cocoons were degummed in a Na2CO3 solution with 1 g l−1 for 1 h at about 85 °C, in order to acquire net silk fibroin fibers, after a certain period of time, replace the old solution with new Na2CO3 solution. Next, dry the silk fibroin fibers in drying oven and dissolve fibers in mixed solution of CH3CH2OH:CaCl2:H2O with molar ratio of 2:1:8 until total dissolution to solution. The silk fibroin solution was dialyzed for 3 days with the distilled water changed three times each day. Finally, the silk fibroin solution was freeze dried.

2.3. Preparation of PVA/silk fibroin hydrogels
Firstly, the PVA solution with a mass ratio of 8% was provided. Then silk fibroin powder was added at a PVA:silk fibroin ratio of 80:10 (wt/wt), 80:20 (wt/wt) and 80:30 (wt/wt) to prepare the PVA/silk fibroin solution. The mixed solutions were transferred into mold, followed by six freeze-thaw cycles to prepare PVA/10%silk, PVA/20%silk and PVA/30%silk composite hydrogels. For PVA/20% silk composite hydrogels, one, two, or six freeze-thaw cycles were adopted to produce PVA/silk fibroin composite hydrogels designated as PS1, PS2, and PS6, respectively.

2.4. Methods
2.4.1. Scanning electron microscopy (SEM)
The hydrogel was frozen and cracked in liquid nitrogen. The cracked surface was sputtered with gold and then scanned under the acceleration voltage of 15 kV by TM-1000 scanning electron microscope (Hitachi, Tokyo, Japan).
2.4.2. Mechanical test
Hydrogels (about 8 mm in diameter and 8 mm in height) were tested using the Instron 5567 mechanical testing machine (Instron, Boston, USA). Six parallel samples were tested for each group of hydrogels ($n = 6$). The crosshead speed was about 1 mm min$^{-1}$. To avoid damaging the instrument, the compression limit was set as a 98% strain.

2.4.3. Swelling behavior of hydrogels
Distilled water was chosen as solution to evaluate swelling behavior of hydrogels. The weight of hydrogel after freeze drying was the initial weight marked as $M_0$, the weight of swollen hydrogel was named as $M_t$. Six parallel samples were tested for each group of hydrogels ($n = 6$). The weight of hydrogel was recorded at each set time until equilibrium. The swelling ratio was calculated as the mass ratio of the net liquid uptake to the dry hydrogel:

$$\text{Swelling ratio} (%) = \frac{M_t - M_0}{M_0} \times 100\%$$

2.5. Cell attachment and proliferation on PVA/silk fibroin hydrogels
Bone marrow mesenchymal stem cells were harvested from vertebrae of rabbits, smooth muscle cells were obtained from the esophagus of rabbits, and BV2 microglial cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (China). The stem cells were cultured in RPMI 1640 medium (Hyclone), and muscle cells and BV2 cells were cultured in high glucose DMEM (Hyclone). Cells were grown in nutrition medium, which contains fetal bovine serum (10%), penicillin and streptomycin (1%). The culture medium was replaced every 2 days. Cells with Passage 3 were chosen for the following experiments. Hydrogels with 8 mm diameter sheet were sterilized by ethanol and sterile PBS. The density of cells was $1 \times 10^5$ cells ml$^{-1}$. After certain times, hydrogel sheets were collected and washed with PBS, and then fixed with glutaraldehyde. Next, washing hydrogel samples with PBS, destroying cell membrane with Triton X-100 (0.1%) and blocking the non-specific protein with bovine serum albumin (1%). Then, cell cytoplasm was stained with phalloidin-FITC ($50 \mu$g ml$^{-1}$, Invitrogen) for about 1 h and washing with PBS in order to eliminate unbound phalloidin conjugate. The cell nucleus was stained with 4, 6-diamidino-2-phenylindole ($10 \mu$g ml$^{-1}$, DAPI, Invitrogen). Finally, after washing with PBS, the cells were imaged by confocal laser scanning microscopy (CLSM) (TCS SP5 II; Leica, Braunschweig, Germany).

2.5.1. Cell proliferation assay
Cells were quantitatively evaluated by the CCK-8 method. Six parallel samples were tested for each group of hydrogels ($n = 6$). Firstly, discarding waste cell culture medium and still keeping the samples at the bottom of cell culture plates, then adding fresh culture medium (300 $\mu$l) and CCK-8 solution (30 $\mu$l), the volume ratio was 10:1, and make sure that samples were completely immersed in the mixed solution, and incubation solution for 4 h in a CO$_2$ incubator. Finally, 200 $\mu$l of the reaction solution was transferred to a 96-well plate. The wavelength of 450 nm was chosen as the maximum absorption wavelength. The test instrument was microplate reader (SpectraMax 190; Molecular Devices, USA).

2.6. Statistical analysis
We express the data by the mean $\pm$ standard deviation (SD). The statistical significance of the differences among the groups was analyzed by one-way ANOVA and then by post-hoc analysis. Significance was considered at a value of $^{* * *}$ $p < 0.01$, $^{* *}$ $p < 0.001$.

3. Results
PVA-based hydrogels were fabricated using a cyclic freeze-thaw method. The structure and properties of hydrogels were regulated by changing the number of freeze-thaw cycles. For example, we investigated the mechanical strength of PVA hydrogels prepared with different freeze-thaw cycles. The results showed that the compressive strength of PVA hydrogels was significantly increased with the increase in cycle number from one to four. However, equilibrium behavior was observed after cycle six (figure S1 is available online at stacks.iop.org/MRX/7/015413/mmedia). Therefore, we choose six as the maximum number of cycles.

Silk fibroin was introduced to facilitate cell growth on PVA hydrogels. Moreover, we evaluated the optimal content of silk fibroin. As shown in figure 1, different contents of silk fibroin (10% wt, 20% wt, and 30% wt) were mixed with PVA to prepare PVA/10%Silk, PVA/20%Silk, and PVA/30%Silk hydrogels, respectively. Bone marrow mesenchymal stem cells grew on these hydrogels after culture for 1, 3, and 7 days, respectively. The results showed that few cells grew on a PVA only hydrogel. After adding silk fibroin, the number of cells adhering
to the hydrogel was increased significantly. When the content of silk fibroin was 20% wt, the number of cells was the largest. Therefore, we adopted the PVA/20%Silk hydrogel for evaluation.

As shown in figure 2, PVA and PVA/20%Silk hydrogels were prepared with freeze-thaw cycles from one to six. The elastic modulus intervals of PVA hydrogels were from 8.7 ± 0.3 kPa (one cycle) to 145.1 ± 7.8 kPa (six cycles), and those of PVA/20%Silk hydrogels were from 14.6 ± 1.3 kPa (one cycle) to 161.0 ± 10.8 kPa (six cycles). For PVA hydrogel, with the increase of freeze-thaw cycles, the elastic modulus of PVA increased gradually, and the modulus of PVA hydrogel with six cycles was about 16.7 times of that with one cycle. In addition, the elastic modulus of PVA was slightly increased after adding silk fibroin.

Figure 1. CLSM fluorescence images of bone marrow mesenchymal stem cells after culture for 1, 3, and 7 days on the surface of PVA, PVA/10wt%Silk, PVA/20wt%Silk, and PVA/30wt%Silk hydrogels and staining with FITC-phalloidin (cytoskeleton, green). Scale bars represent 200 μm.

Figure 2. Elastic moduli of PVA and PVA/silk hydrogels with freeze-thaw cycles ranging from one to six.
By comparing data in figures S1 and 2, we chose PVA/20%Silk hydrogels with one, two, and six cycles (named as PS1, PS2, and PS6, respectively) to maintain a certain mechanical interval. The prepared PS hydrogels possessed a three-dimensional porous network structure as shown in figure 3. The pore sizes of hydrogels were decreased gradually with the increase in cycle number. In addition, an orientation of the pore structure gradually appeared by increasing the cycle number. The PS2 hydrogel began to present a certain orientated distribution, and the PS6 hydrogel was oriented completely. The microstructure of hydrogels can also influence its mechanical properties. Figure 4 shows the different elastic modulus data obtained for PS1 (14.6 ± 1.25 kPa), PS2 (43.3 ± 2.9 kPa), and PS6 (161.0 ± 10.8 kPa) samples. The elastic moduli of PS2 and PS6 hydrogels were about 3- and 11-fold higher than that of the PS1 hydrogel. The swelling ratio of prepared hydrogels was also evaluated, as shown in figure 5(A). The results showed that hydrogels had a highest swelling ratio with one cycle, and the swelling ratio decreased with the increase in cycle number. Furthermore, we chose six freeze-thaw cycles to demonstrate that silk fibroin had little effect on the swelling behavior of PVA hydrogels (figure 5(B)). It was clearly shown that with the increase in freeze-thaw cycles for PS hydrogel preparation, an aligned pore structure had formed and substrate stiffness increased, which decreased the swelling ratio.

In the current study, three kinds of cells—bone marrow mesenchymal stem cells, smooth muscle cells, and BV2 microglial cells—were selected as model cells. The growth behavior of the three kinds of cells was evaluated.
on PS hydrogels. As shown in figure 6(A), stem cells grew on PS1, PS2, and PS6 hydrogels after culture for 1, 3, and 7 days, respectively. First, only a small number of cells adhered to the surface of these hydrogels, and the number of cells did not increase with the prolongation of culture time. In addition, the morphology of stem cells changed dramatically from long shuttles to round. Quantitative results of stem cell proliferation also showed that, with the increase of culture time, stem cells maintained a low absorbance value and did not proliferate (figure 6(B)). The CCK-8 assay results of stem cells were consistent with fluorescence images. The growth behavior of smooth muscle cells presented a similar trend (figure 7). Both fluorescence and CCK-8 results showed that the smooth muscle cells showed little adherence to the hydrogel and hardly proliferated. In particular, the shape of cells also changed from a long shuttle to round. However, BV2 microglial cells as an immune cell type in the central nervous system showed distinct growth behavior compared with the above two cell types (figure 8). BV2 microglial cells were round, and PS hydrogels were beneficial for cell adhesion and growth as shown by fluorescence analysis. In addition, the number of adherent cells was increased with the increase in freeze-thaw cycles. As shown in figure 8(B), the CCK-8 assay results demonstrated that there were few BV2 microglial cells on hydrogels at day 1, and the number of cells increased dramatically at days 3 and 5. Compared with PS1 and PS2 hydrogels, the proliferation of BV2 cells on the surface of PS6 hydrogels was the most obvious. Moreover, the growth trajectory of BV2 microglial cells was consistent with the pore walls of hydrogels. In addition, after 3 days of cell culture, the cell migration depth in different hydrogels was evaluated through three dimensional fluorescence images and calculated using ImageJ software (figure 9). The migration depth of stem cells in PS1, PS2, PS6 hydrogels were 83.3 μm, 126.4 μm, 155.6 μm respectively, the migration depth of muscle cells were 79.2 μm, 81.9 μm, 236.1 μm respectively, the migration depth of BV2 cells were 194.4 μm, 125.0 μm, 263.9 μm respectively. It could be found that the BV2 cells migrated deeper compared with the stem cells and muscle cells.
4. Discussion

To maintain the original structure and properties of PVA and enhance its biofunctions, we introduced silk fibroin into PVA hydrogels and fabricated PVA/silk composite hydrogels. By increasing the freeze-thaw cycle number (i.e. from one to six), the overall initial degree of crystallinity remains unchanged, but reinforcing existing crystals in PVA based hydrogels, so the substrate modulus was enhanced markedly and the pore size and swelling ratio of hydrogels were decreased [15]. The results also showed that silk fibroin had little effect on the mechanical and swelling properties of PVA hydrogels. However, different cell types exhibited various growth states on PVA/Silk hydrogels. For stem cells and smooth muscle cells, their initial shapes were a spindle before seeding on PVA/silk hydrogels. The shape of these two kinds of cells shrank to a circle after culture for 1, 3, and 7 days (figure 6 and 7). This phenomenon suggested that long-shaped cells did not grow efficiently on PVA/silk hydrogels, although some cells adhered to the surface of hydrogels on the first day. However, the cells remained round and did not proliferate with the increase in culture time. For BV2 microglial cells, their original shapes

![Figure 6](image)

**Figure 6.** (A) CLSM fluorescence images of bone marrow mesenchymal stem cells after culture for 1, 3, and 7 days on the surface of PS1, PS2, and PS6 hydrogels and staining with FITC-phalloidin (cytoskeleton, green). Scale bars represent 200 μm. (B) Proliferation of bone marrow mesenchymal stem cells cultured on the surface of PS1, PS2, and PS6 hydrogels measured by the CCK-8 assay.
were round before seeding on PVA/silk hydrogels. These cells not only maintained a round shape, but also a large number of cells had adhered to the hydrogel at day 1. With the increase of culture time at days 3 and 5, BV2 microglial cells had proliferated significantly. Using the PS6 hydrogel (figure 8(B)), the optical density values of cells cultured on the hydrogel for 3 and 5 days was 9.8- and 11.6-fold higher than that of cells cultured on the hydrogel for 1 day. Therefore, the PVA/silk hydrogel system is suitable for culturing BV2 microglial cells, but not suitable for culturing bone marrow mesenchymal stem cells and smooth muscle cells. PVA only hydrogels possess low cell adhesiveness. Therefore, active components are usually introduced into PVA hydrogels through a physical or chemical process to facilitate stem cell growth and differentiation. Such active components include hyaluronic acid [13], chitosan [16], gelatin [17], oligopeptides [18], and karaya gum [19]. For example, when preparing PVA/chitosan composite hydrogels, increasing the proportion of PVA from 80 wt% to 90 wt% changes the stem cell shape from polygonal to spherical [16]. This change in cell morphology on the PVA/chitosan composite hydrogel was consistent with that on our PVA/silk hydrogel (PVA 80 wt%). Therefore, the content of silk fibroin in PVA hydrogel may be one of the factors that affect cell morphology.

Figure 7. (A) CLSM fluorescence images of smooth muscle cells after culture for 1, 3, and 7 days on the surface of PS1, PS2, and PS6 hydrogels and staining with FITC-phalloidin (cytoskeleton, green). Scale bars represent 200 μm. (B) Proliferation of smooth muscle cells cultured on the surface of PS1, PS2, and PS6 hydrogels measured by the CCK-8 assay.
Although silk fibroin hydrogels are beneficial for stem cell growth and differentiation [20, 21], and silk fibroin composite scaffolds are also widely studied, such as silk fibroin/chitosan/graphene oxide scaffolds [22] and silk fibroin/chitosan/bioactive glass nanoparticle scaffolds [23]. These studies indicated that the composition and content of silk fibroin in composite scaffolds play an important role in cell morphology and behavior. Another example was about PVA/BCP (biphasic calcium phosphate) scaffold. When the content of BCP was 70 wt%, bone marrow mesenchymal stem cells presented the state of spreading and stretching, cell proliferation assay showed that with the increase of BCP content from 50 wt% to 80 wt%, the cell proliferation rate increased gradually [24]. In addition, the difference in topology is more than likely to affect cell adhesion and attachment. Pramanik et al [25] reported stem cells grew on PVA/silk fibroin and PVA/silk fibroin/bioactive glass electrospinning scaffold, a small number of stem cells could adhere to PVA/silk fibroin scaffold and spread, more cells could adhere to PVA/silk fibroin/bioactive glass scaffold and showed greater extent of spreading. The results demonstrated the composition of bioactive glass played an important role in the adhesion and spreading.

Figure 8. (A) CLSM fluorescence images of BV2 microglial cells after culture for 3 days on PS1, PS2, and PS6 hydrogels and staining with DAPI (nucleus, blue) and FITC-phalloidin (cytoskeleton, green). Scale bars represent 200 μm. (B) Proliferation of BV2 cells cultured on the surface of PS1, PS2, and PS6 hydrogels measured by the CCK-8 assay (**$p < 0.01$).
of stem cells. Compared with our research results, porous structure in our study and spinning structure from Pramanik’s research have different effects on the growth behavior of stem cells, which can respectively make cells appear round or spindle shaped morphology. Therefore, although the same components of PVA and silk fibroin, the different topological structures of scaffolds could regulate grow behavior of stem cells. Finally, we think that the growth behavior of cells is affected by many factors such as the composition, content of materials and topological structure of scaffolds. Of course, moduli of scaffolds are also very important aspect to regulate cell behaviors [26], however, the moduli of hydrogels did not seem to play a major role in our study. In addition, because of the same composition (PVA and silk fibroin) and content (PVA 80 wt% and silk fibroin 20 wt%) for PS1, PS2, and PS6 hydrogels, so the topological structure of hydrogels may be the main factor regulating cell growth behavior. Although PVA/silk fibroin hydrogels have been used for artificial auricular cartilage [27] and skin keratinocytes [28]. However, to our knowledge, there is almost no research on regulation of the behavior of three kinds of cells by PVA/silk fibroin hydrogels system. Hence, we fabricated a PVA/silk fibroin hydrogel system and explored its regulating effects on bone marrow mesenchymal stem cells, smooth muscle cells, and BV2 microglial cells. As a result, the system inhibited the growth of stem cells and muscle cells, and increased the attachment and proliferation of BV2 microglial cells. If this system has universal applicability, it can inhibit the growth of spindle cells and promote the growth of round or oval cells simultaneously. For example, in cardiovascular disease, this system would be very useful by simultaneously inhibiting smooth muscle cell proliferation and promoting endothelial cell growth. Therefore, we will verify the universal applicability of this system in a future study.

5. Conclusion

In summary, PVA/silk fibroin composite hydrogels were fabricated by a facile cyclic freeze-thaw method without introducing any additives or specific devices. With the increase of freeze-thaw cycles, the pore size of hydrogels was decreased, the elastic modulus was increased, and the swelling rate was decreased. PVA/silk fibroin composite hydrogels inhibited the growth of bone marrow mesenchymal stem cells and smooth muscle cells, and changed their shape from spindle to round. In addition, the composite hydrogel promoted the growth of BV2 microglial cells and maintained their original shape. Furthermore, for BV2 microglia cells, hydrogels with high freeze-thaw cycles (PS6 hydrogels) were more conducive for cell proliferation compared with low freeze-thaw cycles (PS1 and PS2 hydrogels). Hence, PVA/silk fibroin composite hydrogels can be used to regulate cell behavior in the biomedical field.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81471797 and 81701790), the Major Project of 2025 Sci&Tech Innovation (2018B10052), the Ningbo Health Branding Subject Fund (PPXK2018-02), the Natural Science Foundation of Zhejiang Province (LY20C100001), the Zhejiang Key
Laboratory of Pathophysiology (No. 201906), the Natural Science Foundation of Ningbo (2019A610199, 2019A610185), the Ningbo University Fund (XYL19022) and the Open Research Funds of Fudan University (K2019-08). This work was also sponsored by K C Wang Magna/Education Fund of Ningbo University.

Conflicts of interest

The authors declare no conflicts of interest in this work.

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