Photocrosslinked Poly(vinyl alcohol) Nanofibrous Scaffolds

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Biocompatible photocrosslinked poly(vinyl alcohol) nanofibrous Scaffolds were successfully prepared by electrospinning and consequent photopolymerization. The nanofibrous membranes were subjected to detailed analysis by scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and X-ray diffraction (XRD). SEM images showed that morphology and diameter of the nanofibers were mainly affected by degree of substitute of metharyloyl group. XRD and DSC demonstrated form of a larger fraction of amorphous phase due to destroyed hydrogen bonding through methacrylation. Water stability test showed that the nanofibrous membranes improved its water stability with crosslinking degree increased. The indirect cytotoxicity assessments of the nanofibers were studied. And the result indicated the nanofibers was nontoxic to the L929 cells. This novel electrospun matrix would be used as potential wound dressing for skin regeneration.

Keywords: electrospinning, poly(vinyl alcohol), photopolymerization, wound dressing

1. Introduction

Serious skin damage such as full-thickness burns or deep ulcers always needs skin grafts. However, many skin substitutes for grafts have been restrictively employed due to their disadvantages such as high cost, the limited availability of self skin grafts, and problems of immune response and disease transmission [1-3]. To deal with it, many tissue engineered skin substitutes get developed, among which nanofiber matrices have shown tremendous promise, attributed to their unique properties such as oxygen-permeable high porosity, variable pore-size distribution, high surface to volume ratio and more importantly, morphological similarity to natural extracellular matrix in skin, which promote cell adhesion migration and proliferation [4-6]. As a simple and versatile technique to fabricate these ultrafine fibers, electrospinning, have made many polymers including synthetic and natural polymers directly into nanofibers as potential wound dressing [7-9]. Among these polymers, poly(vinyl alcohol) (PVA) is considered as the first investigated and commonly used polymers for nanofiber formation due to its desirable properties, such as nontoxicity, biocompatibility, and especially excellent electrospinnability [10].

Recently, PVA or PVA-based nanofibers have been successfully electrospun from PVA solution [11] or PVA solutions blended with hydroxyethyl cellulose [12], chitosan [13], gum tragacanth [14] or alginate [15]. However, these electrospun nanofibers based on PVA, were usually limited as direct wound dressings because of their dissolution when contact with tissue fluids, which did not protect wounds from environment. So, it is necessary to get stabilized PVA nanofibers by some methods, such as glutaraldehyde...
crosslinking [12, 13] or heat treatment [16]. However, glutaraldehyde has potential toxicity to harm wounded human skin or tissue [17], and heat treatment could damage the biological enzyme or cells immobilized by nanofibers [18].

To overcome these problems, in this research, methacrylated poly (vinyl alcohol) was synthesized containing methacryloyl group and thus photocrosslinked MPVA electrospun nanofibers were prepared with UV irradiation. Here, electrospinning carried out in neutral pH and further photocrosslinking could not only avoid the trace presence of the toxic solvent or crosslinking agent, but also result in the fabrication of functional fibrous biomedical products containing thermal-instability protein drugs or cells. The morphological characterization, crystallization and water resistance were investigated. The potential use of this as-spun fiber mat as a scaffolding material for skin was evaluated in vitro with mouse fibroblasts (L929).

2. Experimental

2.1. Materials

Poly(vinyl alcohol) (PVA, 88% hydrolyzed) was obtained from Kuraray Co., Ltd., Japan. Glycidyl Methacrylate (GMA) was supplied by Adamas Reagent Co., Ltd. 4-Dimethylaminopyridine (DMAP) was purchased by Sinopharm Chemical Reagent Co, Ltd. Darocur 2959 (D-2959, 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propa none) was obtained from Ciba-Geigy Chemical Co. (Tom River, NJ). Mouse fibroblasts (L929) were obtained from Wuhan Beinglay Biological Technology Co., Ltd., China. Dulbecco's modified eagle medium (DMEM), 1% Penicillin-streptomycin, trypsin, 10% fetal bovine serum (FBS), MTT powder was supplied by Shanghai kayon Biological Technology Co., Ltd., China. Other reagents were all A.R. grade.

2.2. Synthesis of methacrylated poly(vinyl alcohol) (MPVA)

MPVA was prepared according to the method reported by Hennink et al [19]. Briefly, 5.0 g PVA was dissolved in 100 mL dimethyl sulfoxide (DMSO) and DMAP was added to it at 1.0 mol % relative to the hydroxyl group of PVA. The required amount of GMA was added in molar ratio to hydroxyl group of PVA and the reaction mixture was stirred for 6 h at 60 °C (Table 1). The mixture was precipitated by acetone and then dried under vacuum for 2 days and stored at -5 °C in the dark. $^1$H NMR spectrum was recorded on a Bruker AV 400 NMR instrument.

Table 1. Mole ratio of reactant and degree of substitute of MPVAs.

| Samples | PVA (g) | Epoxy/OH | DS $^{[a]}$ |
|---------|---------|----------|-------------|
| MPVA1  | 5       | 0.025    | 0.01        |
| MPVA2  | 5       | 0.050    | 0.03        |
| MPVA3  | 5       | 0.100    | 0.07        |

$^{[a]}$ DS was calculated from $^1$HNMR studies.

2.3. Preparation of MPVA solutions

A 10% (w/v) MPVA solution was prepared by 10 g MPVA dissolved in distilled water. For further photopolymerization, a 10% (w/v) MPVA solution was prepared containing the photoinitiator D-2959 (0.1 w/v%). D-2959 was used in this study, as it has been demonstrated to be the least cytotoxic to various cells [20].

2.4. Preparation of nanofiber mats

The electrospinning was performed at room temperature. The 10% MPVA solution or the 10% MPVA solution containing D-2959 was placed into a plastic syringe (20 mL) with a metal capillary having an inner diameter of 0.57 mm. The positive electrode of a high voltage power supply (EST705, Beijing Huajinghui Technology Co., Ltd., China) was connected to the metal capillary by copper wires. The voltage was 25 kV, and tip-to-collector distance was fixed at 12 cm. And the solution feed-rate of 1.0 mL/h was maintained using a syringe pump. A grounded aluminum foil was used as the collector.

2.5. Photocrosslinking of nanofiber mats

The nanofiber mats were irradiated directly under 250 W Hg lamp at exposure intensity of 30 mW/cm$^2$. Upon UV irradiation, the photoinitiator in the mats initiated further crosslinking of MPVA nanofiber mats. The crosslinked nanofibrous nonwoven mats were collected and dried at room temperature in vacuum for 12 h.
2.6. Scanning Electron Microscopy (SEM)

The morphology and diameter of nanofibrous mats were determined by scanning electron microscope (JSM-6510, JEOL Ltd., Japan) at accelerating voltage of 10 kV. The diameters of nanofibers were measured by using image analyzer. At least thirty fibers were statistic in image.

2.7. Differential Scanning Calorimetry (DSC)

The thermal analysis of the electrospinning fibers was studied by using Differential Scanning Calorimetry (DSC822e, Mettler Toledo, Switzerland). Samples sealed in aluminum pans were heated from room temperature to 230 °C at a heating rate of 40 °C/min under 50 mL/min of nitrogen flow, and then quenching to -50 °C. After that, the samples were heated again to 220 °C at a heating rate of 10 °C/min under 50 mL/min of nitrogen flow.

2.8. X-Ray Diffraction (XRD)

The XRD patterns of the electrospinning fibers were performed via X-ray diffractometer (XRD2000, Shimadzu, Japan) with Cu Kα characteristic radiation (wavelength $\lambda=0.154$ nm at 40 kV, 50 mA, and scan speed of 1°/min in the 2θ range of 5-60°).

2.9. Water resistance of crosslinked MPVAs nanofibrous mats

The crosslinked MPVA nanofibrous mats were immersed in deionized water at 37 °C for six hours by changing the water every hour. Finally, the cross-linked mats were lyophilized for observation of morphology changes of crossklinked nanofibers by SEM.

2.10. Cytotoxicity assays

Extracts were prepared from crosslinked MPVA nanofibrous mats by adding fragments of the sterile samples to the culture medium at a concentration of 6.0 cm$^2$/mL and incubating at 37 °C for 24 h without shaking. After this period, the medium was obtained and the crosslinked MPVA nanofibrous mats were removed.

According to ISO-10993, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to determine the extracts toxicity [21]. Initially, 200 μL of L929 cell suspension at density of 105 cells per well were seeded in wells of 96–well plate and cultured for 24 h at 37 °C. After this period, the culture medium was removed and replaced with 100 μL of as-prepared extraction medium. After 24 h at 37 °C, the extracts were removed and 10 μL of MTT solution was added to each well. After 4 h incubation at 37 °C, 150 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. The dissolved solution was swirled homogeneously for about 10 min by the shaker. The optical density of the formazan solution was detected by an ELISA reader (Multiscan MK3, Labsystem Co. Finland) at 490 nm.

For reference purposes, cells were seeded to a fresh culture medium, which was as negative control. Results are depicted as mean ± standard deviation. Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values p<0.05 were considered significant (n= 8).

3. Results and Discussion

3.1. Synthesis of MPVAs

In order to produce a photocrosslinkable product, methacrylation was carried out on PVA molecule chain so that they can undergo free radical polymerization. The insertion of methacryloyl group was always by transesterification under catalyzation by DMPA in DMSO, as indicated by the literature [22]. Three MPVA samples (denoted MPVA1–3) were prepared by using increasing amounts of GMA in order to yield products with different degrees of substitution (DS), the values for which are also listed in Table 1. DS increases from MPVA1–3 indicating that DS is positively correlated to the ratio of GMA used in the reaction. $^1$H NMR of MPVAs and PVA are provided in Fig. 1. Methacrylation is achieved, as evidenced by peaks arising at 5.6 and 6.0 ppm due to protons on the vinyl carbon and a peak at 1.9 ppm due to the methyl group in methacryloyl group. The DS of methacryloyl groups onto the PVA backbone was calculated from the relative integrations of the vinyl protons at 5.6 ppm with respect to the methyl protons of $-\text{COCH}_3$ at 2.0 ppm: $\text{DS} = 3 \times 0.12 \times I_{5.6}/I_{2.0}$. By calculation, DS increased from 0.01 to...
0.07 with mole ratio of epoxy group of GMA to -OH of PVA increased. Other peaks were also attributed (seen in Fig. 1). The solubility of the product in water was evaluated from the visual observation method. As shown in Fig. 2, MPVA could dissolve completely in water at concentration of 10% (w/v), however MPVA solution became milky solution from clear solution with DS increased from 0.01 to 0.07, which indicated that introduction of hydrophobic methacryloyl groups changed the nature hydrophilicity of PVA. In the further experiment, we found PVA could not dissolve in water any more when DS exceeded to 0.12, and water was an antisolvent at time.

3.2. Scanning Electron Microscopy (SEM)

Based on the research findings by us earlier [23], the PVA solution at the concentration above 9% was suitable to form uniform nanofibrous mats by electrospinning. So here, the concentration of 10% are chosen to electrospinning for MPVA. SEM micrographs of the nanofibers (with UV irradiation) obtained at different DS are shown in Fig. 3. No formation of "tailed" micro- and nanoparticles or a bead-on-string morphology was observed. Instead, ultrafine fibers were formed. It is reported that PVA is easy to produce electrospun nanofibrous mat due to its good fiber-forming [24]. The fibers morphology changes were not obvious with different DS or thus different crosslinking density, which indicated that low degree of substitution of methacryloyl group to -OH of PVA did not destroy backbone structure of PVA, leading to reduce of spinnability of PVA.

Correspondingly, the diameter distribution of the nanofibrous mats is presented in Fig. 4. As the DS increased from 0.01 to 0.07 in MPVA, the average diameter of nanofiber mats gradually increased from 487 to 717 nm and the distribution became slightly broader. It was reported that, the evaporation rate of solvent as an important factor to control the terminal fiber diameter [25]. Here, introduction of hydrophobic methacryloyl groups reduced hydrophilicity of PVA, and thus more water molecules were not bonded closely with -OH of PVA with DS increased, leading to rapid increase of the rate of water evaporation during electrospinning, which consequently also caused a rapid increase of polymer viscosity, which reduced the voltage induced stretching of polymer chain, resulting in the formation of wider fibers [26]. In other words, MPVA jet solidified sooner after it came out of the needle, and therefore exposed to voltage induced stretching for a shorter time only, leading to formation of
wider fibers, with DS increased.

3.3. X-Ray Diffraction (XRD)

Figure 5 presents XRD patterns of PVA powder, MPVA nanofibers and MPVA2 after UV photopolymerization. For the pure PVA powder (Fig. 5a), there were four typical peaks at 2θ = 11.3°, 19.3°, 22.5° and 40.6°, similar to the literature [27, 28]. However, for all MPVA nanofibers, the crystalline peak at 2θ = 11.3°, 22.5° and 40.6° disappeared almost and strong peak at 2θ = 19.3° became a relative obtuse and broad. The reason was that, one, the number of hydrogen bonding in PVA was destroyed through methacrylation, thus forming a smaller fraction of crystalline phase and a relatively larger fraction of amorphous phase. The other, subsequent electrospinning retarded the crystallization process of MPVA, which did not lead to the development of the crystalline microstructure of electrospun fibers, due to the stretched molecular chains of the fiber solidified rapidly at high elongation rates during the electrospinning process [29]. After UV photopolymerization, the intensity of peak around 20° in MPVA2 nanofiber was relatively higher and sharper as compared to uncrosslinking MPVA2 (Fig. 5c) showing the presence of relatively higher crystallinity, which was mainly due to the formation of more stable organized structure and increased hydrogen bonding interaction between the chains.

3.4. Differential Scanning Calorimetry (DSC)

DSC curves of PVA, MPVA and nanofibers are reported in Fig. 6. The pure PVA powder displayed a relatively large and sharp endothermic curve with a melting peak (Tm) at 181.1 °C and a glass transition temperature (Tg) at 71.1 °C, respectively (Fig. 6d). However, the endothermic curves of MPVAs became obtuse and broad, and the melting peak shifted from 161.5 °C towards lower temperatures (120.6 °C) with DS increased. Also, Tg of MPVA decreased slightly from 68.9 °C to 68.1 °C with DS increased, lower than Tg of PVA. These results indicated that introduction of methacryloyl group reduced interaction forces of intra/intermolecular hydrogen bonding, leading to easier motion of molecule chain. As seen in Fig. 6b, there was no obvious different in Tg or Tm peaks when MPVA2 was electrospun into MPVA2 nanofibers. However, after photopolymerization, Tm of MPVA2 nanofibers increased from 143.2 °C to 159.8 °C and the peaks became relatively broad and sharp, which indicated that photocrosslinking could efficiently increase the crystallinity of MPVA electrospun nanofibers by increasing hydrogen bonding interaction between the chains and forming more stable organized structure, which was also proved be XRD analysis.

3.5. Water resistance of crosslinked MPVA nanofibrous mats

Dissolution of PVA fiber when contact with water limits its application in biomedical engineering [30]. The MPVA nanofibers without photopolymerization were immediately disintegrated in water due to their high porous surface area, although this behavior of the nanofibers was very advantageous when compared with that of PVA powder which is not water-soluble at room temperature [31]. The morphology changes of MPVA nanofibers with different
DS after UV polymerization upon water immersion were observed by SEM (Fig. 7). It could be seen that, fibrous morphology was almost maintained with little swelling or welding of the fiber junctions (Fig. 7c), which indicated that crosslinked fibers could maintain their structure due to high crosslinking degree. Also, cellular structure in nanofiber mats allows for adequate drainage of exudates and thus reducing the chance of infection, when it was used as wound dressing [32]. However, when crosslinking degree reduced, the morphology gradually showed very rough morphology with convex and concave fibrous shape (Fig. 7b), even if most of nanofibers lost fibrous shape (Fig. 7a). Under UV irradiation, free radical polymerization occurred in metharyloyl group of MPVA to crosslink the PVA molecule chain to form internal network structure, and consequently nanofibers were shown as water-durable.

Fig. 7. SEM micrographs of MPVA nanofibers with different DS after UV polymerization after water immersion at 37 °C for 6 h. (a) MPVA1; (b) MPVA2; (c) MPVA3.

3.6. Cytotoxicity assays

An ideal wound dressing should not release toxic products or produce adverse reactions, which could be evaluated through in vitro cytotoxic tests [33]. In the evaluation, mouse fibroblast cells (L929) were used as reference. Fig. 8 shows indirect cytotoxicity of samples, expressed as a percent of sample absorbance to control absorbance. It could be seen that, no statistically significant differences (p<0.05) were observed in the cell activity of L929 cell within 24 h in the presence of photocrosslinked MPVAs nanofiber mats extracts in comparison with control, which meant that photocrosslinked MPVAs nanofiber mats were nontoxic to L929 cell. All the results indicated that MPVA nanofibers had good biocompatibility, which made them as good wound dressings for skin regeneration.

Fig. 8. Cytotoxicity test of photocrosslinked MPVA nanofibers with negative controls (p <0.05) *p < 0.05 when compared to the negative control of indirect cytotoxicity.

4. Conclusion

In this study, the biocompatible photocrosslinked MPVA nanofibrous scaffolds were successfully prepared by electrospinning and consequent photopolymerization. The photocrosslinked MPVA nanofibrous mat improved its water stability with crosslinking degree increased. Indirect cytotoxicity assessment of the nanofiber mats with mouse fibroblasts indicated that it had good in vitro biocompatibility. These novel electrospun matrices have the potential to be used as materials for wound dressing for skin regeneration.

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