Evaluations of Poly(vinyl alcohol) Hydrogels Cross-linked under γ-Ray Irradiation

Young Chang Nho, Seok-Whan Moon*, Kweon-Haeng Lee**, Chong Won Park***, Tae Suk Suh****, You Jin Jung****, Woong Shick Ahn****, and Heung Jae Chun****†

Korea Atomic Energy Research Institute, Daejeon 305-600, Korea
*Department of Thoracic Surgery,
**Department of Pharmacology,
***Department of Internal Medicine,
****Department of Biomedical Sciences, College of Medicine, Catholic University, Seoul 137-701, Korea

Received November 10, 2004; Accepted December 8, 2004

Abstract: Cross-linked poly(vinyl alcohol) (PVA) hydrogels were prepared under Cobalt gamma ray (Co γ-ray) irradiation. The physical properties of these hydrogels, including their gelation, water absorptivity, and gel strength, were examined to evaluate the applicability of these hydrogels for use as a synthetic extracellular matrix (ECM). The γ-ray-irradiated hydrogels showed improved mechanical properties when compared to glutaraldehyde (GA)-treated hydrogels. The swelling degree was inversely proportional to the gel content and gel strength. The biocompatibility profiles of these hydrogels, for use as tissue engineering scaffolds, have been evaluated (i) in vitro in cultures of mouse fibroblasts and (ii) in vivo by subcutaneous implantation studies in rats. A cytotoxicity assay revealed that the γ-ray-treated hydrogel was approximately half as toxic as the GA-treated PVA hydrogel. Subcutaneous implantation studies in rats showed that GA-treated PVA hydrogels created harsh environments to their surrounding tissue components. However, no severe acute foreign body reactions were found around the γ-ray-treated implants, and these reactions decreased upon increasing the implantation time. These results suggest that Co γ-ray-treated PVA hydrogels are promising materials for the design of matrices for cell growth and proliferation.

Keywords: poly(vinyl alcohol), hydrogels, gamma-ray irradiation, cytotoxicity, tissue compatibility

Introduction

Poly(vinyl alcohol) (PVA) hydrogel has gained popularity as a scaffold for tissue engineering because of its high water content, good biocompatibility, and consistency that is similar to that of soft tissue [1]. PVA hydrogel consists of a three-dimensional network of polymer chains, which may facilitate diffusional exchange of nutrients and waste products with its surrounding environment to allow cell growth. So far, chemical methods using small-molecular-weight cross-linking agents have been employed widely for the preparation of PVA hydrogels [2]. Unfortunately, these cross-linking processes proved to be cytotoxic [3]. Therefore, investigating a new cross-linking method for the preparation of hydrogels that exhibit lower toxicity and high compatibility with cells and tissues desirable in the fast-growing field of tissue engineering. Irradiative cross-linking techniques appear to be good candidates to fulfill such a role [4,5]. Radiation can induce chemical reactions to modify polymers even when they are in the solid state or at a low temperature. In addition, radiation cross-linking can be easily adjusted by controlling the radiation dose, it is reproducible. The finished product contains no residuals of substances required to initiate the chemical cross-linking and that may restrict its application possibilities [6].

The aim of this work was to evaluate PVA hydrogel prepared by Cobalt gamma-ray (Co γ-ray) irradiation for use as a tissue engineering scaffold material. Glutaraldehyde (GA), a widely used chemical cross-linking agent in the preparation of bioprostheses, was used to prepare...
control PVA hydrogel while evaluating the physical properties, cytotoxicity, and tissue compatibilities of γ-ray-treated PVA hydrogels.

Material and Methods

Preparation of Hydrogel
Samples of 10, 15, and 20 w/v (%) PVA (MW: 1.46 × 10³–8.5 × 10⁶, Aldrich) were dissolved in distilled water at 90°C, and then they were placed into a petri dish. Prior to irradiation, the solutions were maintained at room temp for 12–24 h to remove air bubbles, and then they were packed. The aqueous PVA solutions in a petri dish were irradiated with Co γ-rays (2000000 Ci, AECL, Ottawa, Canada) of various doses (10–90 kGy) at room temperature. The solution prepared above was loaded into a Tygon tube 15-cm in length; 1.6-mm in inner diameter using a hypodermal syringe. The ends of tubes were sealed using stainless-steel clip. The tubes containing these solutions were exposed to Co γ-ray irradiation (50 kGy) to prepare rod-type cross-linked hydrogels [4]. Cross-linked PVA samples containing 0.2–1% (w/v) GA were prepared as described in a previous paper for use as a positive control [7]. The GA-treated samples were rinsed for 4 h in phosphate-buffered saline to eliminate residual GA.

Gelation Content
The hydrogels formed by irradiation were immersed in distilled water for 48–72 h at room temperature until the gel reached its equilibrium state; it was then removed from the distilled water. Water on the surface of the swollen gels was removed with the aid of filter paper. The gels were then dried in vacuum at 80°C for ca. 48 h until they reached constant weight. The gelation content was measured from the following equation (1). The term \( W_d \) is the dried gel weight and \( W_i \) is the initial weight of the polymer in the polymer solution [8,9].

\[
\text{Gel percent} = \frac{W_d}{W_i} \times 100
\]

Swelling Studies [10]
The degree of swelling can be described as the swelling ratio of the hydrogels. The gel samples were immersed in distilled water for 48 h at room temperature until the swelling of the gel reached its equilibrium state. After the water on the surface of the swollen gels was removed by using filter paper, the weight \( W_s \) was determined. The gels were dried in a convection oven at 80°C until they reached constant weight; the dried gel was removed periodically to ensure that equilibrium was attained. The term \( W_s \) is the weight of the swollen gel and \( W_d \) is the dried gel weight [8,9].

\[
\text{Swelling percent} = \frac{W_s - W_d}{W_d} \times 100
\]

The swelling percent and change of gel strength during swelling were examined. At this time, the hydrogels were immersed in water at 37°C until the hydrogels reached their equilibrium state of swelling.

Gel Strength and Elongation at Break
The mechanical properties of the hydrogels were obtained by determining the gel strength. Gel strength is the peak force \( F_b \) in grams multiplied by the distance \( \Delta D \) to the rupture (measured in centimeters). Tests were conducted by using a “TA-XT2” texture analyzer (SM Co. Ltd., England) at room temperature. The gel was mounted on a test die containing a hole [9].

\[
\text{Gel Strength (g·cm)} = F_b \times \Delta D
\]

Cell Culture
The cytotoxicity of hydrogels was evaluated on mouse fibroblasts. L 929 fibroblasts (ATCC CCL1, NCTC clone 929, from mouse connective tissue) were cultivated in 10% (w/v) FBS-Dulbecco’s modified Eagle medium (DMEM, Sigma). The fibroblasts for monolayer culture were plated at a density of 5 × 10⁶ cells/2 mL in six-well culture dishes, and incubated in a 5% CO₂ incubator at 37°C.

Preparation of Extracts
Each hydrogel (1 g) was immersed in 5 mL of a serum-supplemented cell culture medium. The extracts were prepared in an incubator at 37 ± 1°C for 24 h; high temperatures may cause denaturation of serum proteins [11].

MTT Assay
A monolayer culture of L 929 was initiated at a density of 1 × 10⁴ cells per 96 well, and incubated for 72 h. Following incubation, the cell culture medium was aspirated from the monolayers and replaced with serum-supplemented cell culture medium extracts. The extracts were tested in duplicate at an extraction concentration of 25%. All cultures were incubated for 72 h at 37 ± 1°C in an incubator containing 5 ± 1% carbon dioxide. The amounts of surviving cells after incubation were evaluated by an MTT staining assay.

Subcutaneous Implantation Studies [12]
The rod-type PVA hydrogels that were cross-linked inside Tygon tube were cut into strips (1.5 mm) and inserted into a sterile trocar of 15 G aseptically. SD rat
Results and Discussion

Figure 1 shows the degree of gelation of the hydrogels. The gel content of the hydrogels prepared using GA was in the range 35–75% and it increased with an increase of the GA content. The γ-ray-irradiated hydrogels containing 10 and 15% PVA show very low gel contents at an irradiation dose of 10 kGy. This finding may be attributed to the fact that a radiation dose of 10 kGy was insufficient to form a true gel from a PVA solution. However, increasing the radiation dose surmounted this problem to a certain extent. The degree of swelling of the irradiated hydrogels was in the range 1000–3000% (Figure 2). However, GA-treated hydrogels showed a maximum degree of swelling of ca. 1000. This finding can be attributed to the differences in the crosslinking mechanisms of the two samples. Irradiation with γ-rays on a PVA solution may produce free radicals at the backbone of the PVA chain through dissociation of a hydrogen atom from a tertiary carbon atom. The chains possessing free radicals then bound together by a free radical recombination reaction, resulting in a large space forming between the crosslinked network of chains. In the case of the GA-treated hydrogels, however, there exists a limitation of space between the chains because of the small chain length of GA. At any rate, the degree of swelling of all of the samples decreased as both the radiation dose as well as the GA concentration increased. The swelling percentage was inversely proportional to the gel percentage. The gel strength of the hydrogels is shown in Figure 3. For all samples, the gel strength increased as both their radiation dose and the GA concentration increased. However, compared to GA-treated PVA, the, γ-ray-irradiated PVA hydrogels showed enhanced gel strength at each content of PVA.

Figure 4 shows the MTT reduction in mouse fibroblast cultures after 1, 2, and 3 days of incubation, in the presence of extracts of the γ-ray- and GA-treated samples. After 1 day of incubation, the MTT reduction capacity of the γ-ray-crosslinked hydrogels was 43%, while that
the GA-treated PVA was 20%, i.e., the cytotoxicity induced by the GA-treated PVA sample was almost twice that of the toxicity induced by the γ-ray crosslinked samples. When the incubations were prolonged up to 2 days, the toxicity of the γ-ray-cross-linked samples gradually decreased. In addition, at 3 days, the MTT reduction reached over 90% when compared to the (-) control (serum). On the other hand, the GA-treated hydrogel still exhibited severe cytotoxicity; the MTT reduction was less than 60% when compared to the (-) control, even 3 days of incubation. The obvious cytotoxicity caused by the GA-treated hydrogel can be attributed to the unreacted residual GA that existed in the extract. Glutaraldehyde, a difunctional nucleophilic aldehyde, creates rapid cross-links between aldehyde and amino groups when in contact with proteinous substances [13]. In addition, this reaction can be performed at extremely low concentration of GA (as low as 0.6 g/mL). The creation of this cross-link inhibits collagen synthesis and encourages dystrophic calcification, which in turn continues to be cytotoxic [13]. Figures 5 and 6 show the acute foreign body reactions that occurred during 1 week of implantation. One day after implantation, the γ-ray-treated PVA hydrogel was covered by a large number of acute inflammatory cells, mainly consisting of polymorphonuclear leukocytes (PMNs) and monocytes (Figure 5-A). The surrounding tissue exhibited some degree of edema and congestion. The GA-treated sample showed increased numbers of PMNs, as well as an increased extent of edema and congestion.  

**Figure 4.** MTT reduction capacity values expressed as percentages of control values, obtained by cell incubations (means ± SD, n = 3).

**Figure 5.** Acute inflammatory cells, polys and monocytes, and edema surrounding (A) γ-ray-treated PVA and (B) GA-treated PVA, injected underneath the dermis; 1 day, magnification 10 × 10 (ocular lens x objective lens), H & E.

**Figure 6.** Granulation tissue, newly growing capillaries and fibroblasts, that grew into the interfaces: (A) γ-ray-treated PVA and (B) GA-treated PVA; 1 week, 10 × 20, H & E.
congestion, when compared to the γ-ray-treated PVA (Figure 5-B). One week after implantation, the γ-ray-treated PVA hydrogel was surrounded by a fibroblast capsule that consists of PMNs, monocytes, and infiltrated macrophages with a small number of foreign body giant cells (Figure 6-A). A large number of neo-vascular, capillaries, and venules, as well as fibrosis granulation tissues, were also observed at the interface. Fibrous connections extended to the capsule of the distal radius. The GA-treated sample, on the other hand, exhibited a decreased extent of granulation tissue formation and an increased extent of edema and congestion when compared to the γ-ray-treated PVA (Figure 6-B).

Acute inflammation is of relatively short duration, lasting from a few minutes to a few days. Its main characteristics are the exudation of fluid with plasma proteins (edema) and the emigration of leukocytes (predominantly PMNs) [14]. Although the nature of the initial tissue responses during the first 7 days was similar for both hydrogels used in this study, the amount of exudate and the number of acute inflammatory cells were different. The amount of exudate formation against the implants largely depends on the hydration capacity of the implant. Waring and coworkers suggested that materials having high hydration properties could be beneficial in the treatment of edema by absorption of exudative fluids [15]. PVA is known to possess excellent water swelling properties [7]. Therefore, a small amount of exudate formation around the PVA implant can be expected (Figure 6-A). However, GA-treate PVA exhibited a large extent of edema formation with great numbers of vascular congestions. This finding can be attributed to the fact that some amount of GA was released, and reacted with small fragments of protein at the interface. These fragments in turn may stimulate inflammatory exudate cells, causing an increase in the extent of early edema with increased numbers of acute inflammatory cells [16].

After three weeks of implantation, the γ-ray-treated PVA hydrogel showed an increased extent of fibroblast deposition with a small number of giant cells (Figure 7-A). On the other hand, the GA-treated hydrogel exhibited mild fibrosis with a large group of giant cells (Figure 7-B). Chronic inflammation due to the implantation of biomaterials can be characterized by decreased numbers of PMNs and increased numbers of macrophages, lymphocytes, giant cells, and fibroblasts, which are actively involved in the repair reaction by forming a fibrous tissue. After 3 weeks of implantation, the γ-ray-treated PVA samples exhibited the typical chronic inflammatory responses mentioned above including the final phase tissue reactions characterized by a thick fibrous capsule having small numbers of macrophages. However, the GA-treated PVA still showed the unusual chronic reactions of polymers producing or releasing toxic substances persistently [17].

In conclusion, this study has demonstrated the feasibility of using Co γ-rays to crosslink PVA for a variety of tissue engineering applications. PVA hydrogels treated under Co γ-ray irradiation exhibited greatly improved physical properties and biocompatibility than did a conventional PVA hydrogel fixed using GA. Although the study of the long-term biostabilities is in progress, the present findings may aid the design of polymeric matrices intended to be repopulated by cell in-growth.

Acknowledgments

This work was supported by the Korea Food and Drug Administration Grant: KFDA-04092-TIS-052.

References