

Cytotoxicity Evaluation of Hydrogel Sheet Dressings Fabricated by Gamma Irradiation: Extract and Semi-Direct Contact Tests

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Abstract

Cytotoxicity presents one of the required criteria in the biological evaluation of medical devices. In this study, the semi-direct contact test was used to evaluate the potential cytotoxicity of hydrogel wound dressings compared to the conventional extract test. Three types of hydrogel sheets were fabricated from poly(vinyl alcohol) (PVA) by gamma irradiation: Bare sheets, silver (Ag)-coated sheets, and *Aloe vera* (AV)-coated sheets. In the extract test, L929 cells were cultured in the extract derived from the elution of hydrogel samples. For the semi-direct contact test, the cells were cultured *in situ* with the hydrogel samples placed inside transwell inserts above the cell monolayers. At the endpoint of both tests, MTT assay was performed, and the cell viability was determined from the absorbance of formazan. Only the bare and AV-coated hydrogel sheets showed cell viability above the 70 % threshold that ensured the non-cytotoxicity by both tests. For Ag-coated sheets, less than 70 % cell viability occurred when Ag coating was ≥ 0.2 mg/cm². Interestingly, the formazan-depleted area underneath the Ag-coated sample could be clearly observed by the semi-direct contact test. The release of Ag in the form of nanoparticles was confirmed by UV-Vis absorption at 420 nm. In conclusion, the semi-direct contact test can serve as a reliable alternative to the conventional extract test in evaluating the potential cytotoxicity of hydrogel wound dressings.

Keywords: Hydrogel sheet dressings, Gamma irradiation, MTT assay, Extract test, Semi-direct contact test

Introduction

Hydrogels are 3-dimensional networks of crosslinked hydrophilic polymers that can absorb and contain a large amount of water up to thousands of their original dried weights without being disintegrated [1,2]. The soft consistency of hydrogels enables them to mimic the structure of natural tissue and make them an attractive candidate for a wide range of biomedical applications including contact lenses, wound dressings, drug delivery systems and hygiene products [2,3]. In terms of wound dressings, hydrogels in the form of flat sheets or filler strands can be used as a protective barrier over skin lesions to absorb wound exudate and provide the appropriate moist environment that promotes natural wound healing [4-8].

For medical devices that will be used in contact with the body, cytotoxicity evaluation is listed as one of the required tests for biological effects that medical devices across all categories must comply to according to the International Organization for Standardization (ISO) [9,10]. In addition, cytotoxicity evaluation also presents the initial step in the biocompatible evaluation process of biomaterials or tissue-engineered scaffolds before to the efficacy studies in animals and clinical trials [11]. Three different methodologies including extract, direct contact, and indirect contact tests are listed as guidelines for the cytotoxicity evaluation of medical devices (ISO 10993-5) [12]. In the extract test, cells were cultured in the fluid that has been used to elute the tested materials over the specified period, so that any leachable

toxic substances from the exposed surface of materials are collected in the eluent. By contrast, in the direct contact setup, tested materials are placed in contact *in situ* with cell culture, providing the assay with capability to detect even weak toxicity that may leach out from the materials. Finally, as for the indirect contact test, agar overlay assay or filter diffusion method is used to determine if toxic molecules may diffuse through the agar or cellulose ester filter and reach the cell monolayers underneath. Each of these different test setups present pros and cons in the evaluation of cytotoxicity. Although the extract test is mostly common and widely performed, the elution process to acquire extract media takes extra setup and cells are only exposed to the extract media without being in direct contact with the tested materials as in their actual applications [13]. In terms of agar overlay assay, the leachable toxic materials can be absorbed by agar, resulting in the underestimated cytotoxicity [14], whereas the direct contact test can be overshadowed by several complications such as bacterial contamination and mechanical damage of cells due to pressure or abrasion [15]. To this end, it is of high interest to develop an appropriate method for cytotoxicity testing that provides ease of setup, accurate, reliable and relevant results that represent the actual use of medical devices.

Several modifications have been made to the existing cytotoxic tests. For example, a semi-direct contact test has been developed to allow the tested materials to be incubated *in situ* with cell culture inside tissue-culture well plate without causing any physical damages to the cells [16]. In this setup, a transwell insert or Boyden chamber with the membrane at the bottom serves as a sample holder inside the well. As the sample holder hangs above the cell monolayer, any leachable substances from tested materials can diffuse through the porous membrane and reach the cells underneath, thus avoiding any oppression or scraping of the cells due to sample movement. The use of transwell insert has been adopted to evaluate the cytotoxicity of medical products such as hydrogel sheets and bone tissue engineered scaffolds with comparable results to those obtained from the conventional extract test [17,18].

In this study, gamma irradiation was used to prepare crosslinked bear PVA hydrogel sheets. The bear hydrogel sheets were further modified by coating with silver nitrate and AV for bactericidal and moisturizing effect, respectively. In the extract test, all hydrogel samples were first eluted in culture media, which subsequently served as extracts for cell culture in 96-well plate (**Figure 1(a)**). In the semi-direct contact test, hydrogel samples were placed inside the transwell inserts with 1- μm porous membrane above the cell culture in 12-well plate (**Figure 1(b)**). The MTT assay was used to determine the cell viability in both tests. Therefore, the semi-direct contact test can be assessed as a reliable alternative setup to the conventional extract test in evaluating the cytotoxicity of hydrogel dressings.

Materials and methods

Chemicals and reagents

PVA (M_w 89,000 - 98,000 Da), silver nitrate (AgNO_3), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Minimum Essential Medium (MEM), fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were purchased from Sigma Aldrich (St. Louise, USA). AV leaves were purchased from Prachaukirikan province. Mouse fibroblast cell line, L929 (lot# 62727942), was purchased from ATCC (USA). Polyurethane containing zinc (RM-A) and high-density polyethylene (RM-C) were purchased from Hatano (Japan). Transwells with permeable PET membrane of 1- μm pore size (Falcon 353103) were purchased from Corning (USA). Deionized (DI) water was used in all experiments.

Preparation of bare hydrogel sheets

A 10 % (w/v) PVA solution was prepared by dissolving PVA powder in boiling water for 30 min and then mixed thoroughly by magnetic stirrer for 1 h at 80 °C. After the solution was cooled to room temperature, it was transferred to round Petri dishes with a diameter of 5.5 cm. Each sample was then sealed inside a plastic bag before exposed to gamma irradiation at a dose of 40 kGy at the Gem Irradiation Center facility, Thailand Institute of Nuclear Technology (Public Organization). DI water was used in all experiments.

Surface coating of hydrogel sheets with AV juice and silver nitrate

The stock of lyophilized AV was first prepared from fresh AV leaves and kept frozen at -80 °C. A 40 mg/mL AV solution was freshly prepared before the coating process by reconstituting lyophilized AV powder in DI water. Undissolved hard fibers were removed by centrifugation before a 1 mL drop of AV solution was applied evenly on the surface of the bare hydrogel sheet over the entire circular area of 24 cm^2 .

To modify bare hydrogel sheets with silver (Ag) coating, a stock solution of 40 and 80 mM AgNO₃ was first prepared by dissolving AgNO₃ in water. Then a 127- μ L drop of stock solution was applied evenly over the entire 8 cm² surface area of bare hydrogels to yield a final concentration of 0.1 and 0.2 mg/cm² Ag-coated hydrogel sheets, respectively. After coating, both AV-coated and Ag-coated hydrogel sheet samples were sealed inside plastic bags for sterilization by gamma irradiation at a dose of 25 kGy.

Cell culture

The L929 cells were cultured in MEM supplemented with 10 % FBS, 4 mM L-glutamine, and 100 IU/mL penicillin-streptomycin in a humidified incubator at 5 % CO₂ and 37 °C. This supplemented media will be referred to as complete media. The complete media was replenished daily and subculture was performed at ~ 80 % confluency. Cells within 10 passages after purchased were used in all experiments.

Preparation of sample for extract test

The extract test used in this study followed the guideline outlined in ISO 10993-12 [19]. All samples including bare hydrogels, AV-coated hydrogels, and Ag-coated hydrogels, were cut into 0.5×2.5 cm² sheets. Due to the absence of specific guidelines for estimating the volume of extracts per absorbent materials including hydrogels and hydrocolloids, the ratio of 0.1 g of hydrogel per 1 mL of media was used to elute all hydrogel samples. This particular ratio was adopted from the guideline provided for porous or textile materials. In our experiment, this total volume of media to extract hydrogels was less than what would otherwise be based on surface area calculation. Therefore, our elution procedure could ensure that the extract possessed the highest possible concentration of leachable substances for the subsequent MTT assay. In line with the ISO guideline, an additional volume of fresh media was added to compensate for the volume of the media absorbed by hydrogels.

All samples were fully submerged in complete media inside 15-mL centrifuge tubes and incubated at 37 °C on an orbital shaker at 60 rpm for 72 h. In addition, polyurethane containing zinc (RM-A) and high-density polyethylene (RM-C) (Hatano, Japan) were also eluted in complete media to serve as positive and negative controls, respectively. In terms of blank controls, the same complete media without hydrogels was incubated inside the 15-mL tube. The extracts obtained from hydrogels were used as 100 % concentration and also diluted in complete media to final concentrations of 50, 25 and 10 % (v/v), which were in accordance with 1 logarithmic range dilution specified in ISO-10993-5 [12].

Extract test

The extraction media of all samples including positive, negative, and blank controls were used in this set of experiments. According to ISO 10993-5 protocol, the L929 cells were seeded on 96 well plates at a density of 10⁴ cells per well in 100 μ L of complete media. This seeding density ensured that the cells were still at a growing phase over the entire duration of 48 h before the MTT assay. After incubation at 37 °C in humidified 5 % CO₂ for 24 h, the cell culture media was removed. All wells under columns 2 and 11 were filled with 100 μ L of extraction media obtained from blank control. All wells under columns 3, 4, 5, and 6 were filled with 100 μ L of extraction media obtained from hydrogels or packing plastics at concentrations 100, 50, 25 and 10 %. Finally, wells under columns 7 and 8 were filled with extraction media obtained from positive and negative controls. The cells were then incubated in extract media for another 24 h before MTT assay was performed.

Semi-direct contact test

The semi-direct contact test was adapted from Wang *et al.* [18]. Cells were seeded at a density of 10⁵ cells per well in a 12-well plate and allowed to grow for 24 h at 37 °C in humidified 5 % CO₂. After that bare, AV-coated and Ag-coated hydrogel samples were cut into 0.6×0.6 cm² piece, which covered about 1/10 of the area of the cell monolayer in the well, and then transferred to the insert and fitted inside the well. At the end of 24 h incubation period, inserts were removed and MTT assay was performed.

MTT assay

Cell viability was assessed by measuring the metabolic activity of the cells using MTT assay. The MTT solution was freshly prepared at a concentration of 1 mg/mL in MEM media without phenol red. In some cases, the MTT solution was prepared at most 72 h prior to the experiment and kept frozen at -20 °C. Following the extract test, 50 μ L of MTT solution was added to each well after the removal of extraction media, and the cells were further incubated in MTT solution for 3 h at 37 °C and 5 % CO₂. Similarly, following the semi-direct contact test, 1 mL of MTT solution was added to the cells. In viable cells, MTT can be reduced into formazan crystals inside the cells by mitochondrial dehydrogenase

enzyme. The resulting formazan formed inside the cells then were dissolved in isopropanol and the blue-violet solution was quantified by measuring absorbance at 570 nm on a microplate reader (Molecular Devices). Finally, cell viability was determined based on the ratio of absorbance of each sample over blank controls. Data were collected from 3 independent experiments.

Characterization of released AgNPs from Ag-coated hydrogels

The Ag-coated hydrogels were cut into the same size as those used in the semi-direct contact experiment before incubated in 1 mL of DI water for 24 h. After that, the supernatant was collected to be analyzed by a UV-Vis spectrophotometer (Evolution 300, Thermo Scientific). Measurements were recorded from 3 replicates to confirm the leachable silver nanoparticles (AgNPs) from the Ag-coated hydrogels.

Statistical analysis

Data were presented as the mean \pm standard error of the mean (SEM). Statistical analysis between 2 groups was performed by Student's t-test with $p < 0.05$ (*) indicating statistical significance.

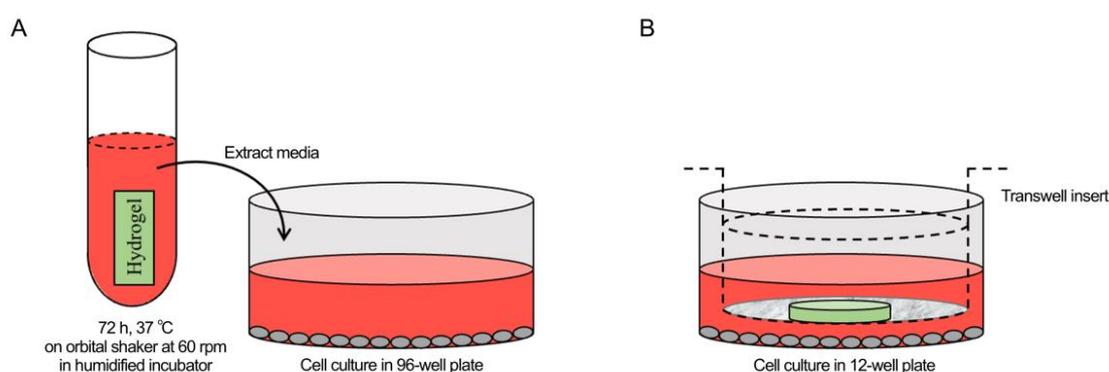


Figure 1 Schematic representation of *in vitro* cytotoxicity evaluation by (A) extract test and (B) semi-direct contact test with a transwell insert.

Results and discussion

Cytocompatibility evaluation of bare hydrogel sheets by extract test

According to the ISO guideline, the extracts eluted from bare hydrogel sheet dressings were used as an original extract at 100 % concentration, and also as dilutions that were adequately spaced within 1 logarithmic range of the original extract at 50, 25 and 10 % (v/v). The viability of cells treated with extracts at all concentrations was within the same range at 87 - 94 % (**Figure 2**). In particular, the percentage of cell viability obtained from 100 % extract was relatively the same as the value obtained from high density PE that served as a standard negative control (**Figure 2**, control (-)). As a comparison, the viability of cells after treatment with extract eluted from commercial hydrogel dressing was 103.40 ± 1.42 %. By contrast, the lowest cell viability of ~ 6 % was obtained from a standard positive control (**Figure 2**, control (+)). As the cell viability of > 70 % (**Figure 2**, dotted line) was indicative of tested materials having non-cytotoxic potential [12], our results indicated that bare hydrogel sheets fabricated by gamma-irradiation was cytocompatible. In addition, as the elution process of hydrogels was performed for the duration of 72 h designated for implants rather than the 24 h period required for skin-contact surface devices [10,19], these results could warrant further *in vivo* evaluation of hydrogels as implants.

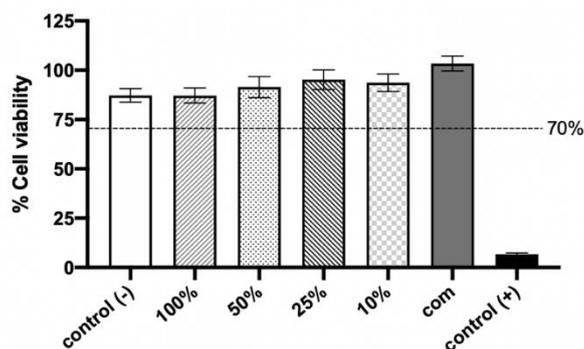


Figure 2 Cytotoxicity evaluation of bare hydrogel sheet dressings using MTT-based extract test compared to commercial dressing (com). Control (-) and control (+) refer to negative and positive standard reference materials.

The fabrication methods, as well as polymers used in the construction of hydrogel, are of equal importance in affecting the biological properties of the resulting hydrogels. In our work, PVA was chosen as the base polymer due to its reported biocompatibility and excellent mechanical properties [2,20]. However, the chemical method used to fabricate PVA into a permanent gel capable of withstanding changes in temperature, ionic strength, and pH can impart some levels of toxicity. For example, glutaraldehyde is widely used as crosslinkers to bridge the molecules of polymers containing hydroxyl or amine groups together [2]. Despite its effectiveness, the crosslinker itself has potential cytotoxicity and can be harmful to the body if not completely removed [21,22]. To this end, crosslinking by the use of high energy radiation such as gamma or electron beam provides an alternative platform to fabricate hydrogels at relatively mild conditions without the use of harmful chemical reagents [1-3]. Previous studies also showed that gamma irradiation could be used to effectively process cytocompatible PVA-based hydrogels containing silk fibroin [4] or silk sericin [23]. Therefore, these cytocompatibility results of bare hydrogel sheets approve both the materials and processing method, providing an important 1st step to warrant a further biological evaluation in an animal model such as wound healing efficacy, or skin irritation patch test in a clinical trial.

Effect of AV and Ag coating on the cytocompatibility of modified hydrogel sheets

To further modify hydrogel sheets with active ingredients, silver nitrate and AV juice were incorporated into hydrogels by surface coating and sterilized by gamma irradiation. Because all bare, AV-coated, and Ag-coated hydrogel sheets will be used in their as-fabricated form in contact with skin, only the original extract at 100 % concentration was used in the MTT assay. **Figure 3** shows the cytotoxicity evaluation of bare and modified hydrogel sheet dressings with commercial dressings embedded with silver nanocrystalline served as a comparison to Ag-coated hydrogels.

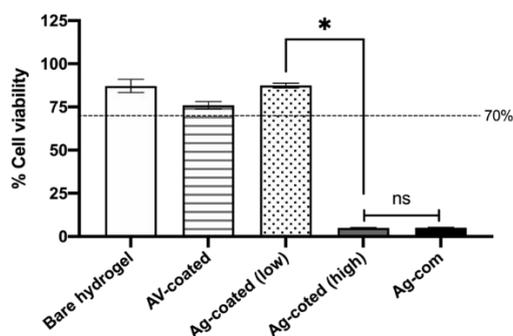


Figure 3 Cytotoxicity evaluation of bare and modified hydrogel sheet dressings using extract test. Hydrogels coated with 0.1 and 0.2 mg/cm² AgNO₃ are denoted as Ag-coated (low) and Ag-coated (high), respectively, and Ag-com refers to commercial dressings with silver nanocrystalline. The symbol ns indicates not significantly different.

The cell viability of bare and AV-coated hydrogels was above 70 %, whereas for Ag-coated hydrogels the cell viability depended on the concentration of AgNO₃ coating. At low concentration of 0.1 mg/cm², Ag-coated hydrogel sheets showed > 70 % cell viability. However, at a higher concentration of 0.2 mg/cm², the cell viability decreased significantly to merely 5 %, which was relatively the same level as commercial dressings embedded with silver nanocrystalline. This extremely low level of cell viability of both our hydrogel sheets and commercial dressings somewhat reflected the high potential of toxicity of silver, especially when in prolonged contact with skin as the samples were eluted for 72 h. By contrast, lower Ag concentration did not perturb the viability of cells to the potential toxicity level despite having the same prolonged elution process. These findings of the hydrogel sheet dressings coated with low Ag concentration were similar to a previous study [24] on polyvinyl pyrrolidone hydrogels embedded with silver nanoparticles.

Evaluation of cytotoxicity using a modified semi-direct contact test

In this method, hydrogel sheet dressings were secured inside transwell inserts and submerged in the culture media above the monolayer of L292 cells, thus mimicking their actual use on the skin while avoiding any possible damage from hydrogel movement on the monolayer. **Figure 4** shows the cytotoxicity evaluation of L292 cells after 24-h incubation in a semi-direct contact condition with bare hydrogels, AV-coated hydrogels, and Ag-coated hydrogel sheets at 0.2 mg/cm².

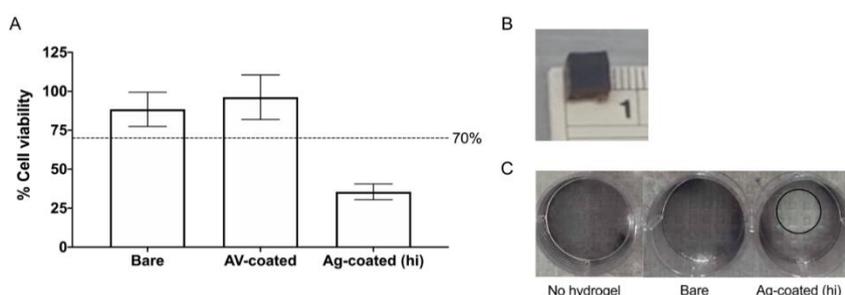


Figure 4 Cytotoxicity evaluation of bare and modified hydrogel sheet dressings by semi-direct contact test. (A) % cell viability, (B) representative image of Ag-coated hydrogel and (C) formazan crystal formation under transwell insert. The circle indicates the formazan-depleted zone observed in Ag-coated hydrogel.

Similar results of cell viability based on different types of hydrogel sheet dressings were also obtained from the semi-direct contact test. The cell viability of bare and AV-coated hydrogels was well above 70 %, while it was reduced to ~ 35 % for Ag-coated hydrogels. Nevertheless, this percentage of cell viability was higher than the value obtained from the extract test (35.5 ± 5.1 vs. 5.0 ± 0.9), which could be due to the different duration of sample incubation. In the semi-direct contact test, Ag-coated hydrogel was incubated *in situ* with cell culture for 24 h, so any leachable Ag from the hydrogel sample would affect the cells during that time. However, in the extract test the sample was incubated for 72 h, thus the cells would experience the effect from the higher amount of leachable Ag presenting in the extract during the 24-h incubation.

The selection of porous membrane that made up transwell plays an important role in determining the potential cytotoxicity of the tested materials. In this study, Ag released from Ag-coated hydrogel sheet presents the leachable substance capable of permeating through the PET membrane having 1- μ m pore size and affecting the cells to the toxicity level. However, a different result was reported in a previous study, which showed that there was little or no toxicity with the transwell insert containing silver hydrogel on normal human fibroblasts [25]. In that study, the transwell inserts with a pore size of 0.45 μ m were used to evaluate the cytotoxicity of wound dressing embedded with 0.84 - 1.34 mg/cm³ silver nanocrystalline. Given the much higher Ag concentration and smaller pore size, this could result in little or no toxicity as the released silver might have been adsorbed onto the membrane and did not reach the cells underneath the insert [26]. Based on these results, the semi-direct contact test can be applicable to other materials than hydrogels used in the fabrication of medical devices. However, the limitation of the test concerns the matching of porous membrane and the size of leachable substances in which the pore size of membrane should surpass the size of leachable substances to avoid false negative results.

Release of AgNPs from Ag-coated hydrogel sheets

The observed cytotoxicity of Ag-coated hydrogels could be attributed to leachable silver in the form of AgNPs from the hydrogels. **Figure 5** shows the UV-Vis spectrum of supernatant collected from Ag-coated hydrogels after incubation in water for 24 h. A noticeable peak was observed at wavelength 420 nm, which corresponded to the characteristic absorption of colloidal AgNPs [7]. Therefore, this ensures the presence of AgNPs that interacted with the cells in both semi-direct contact and extract test.

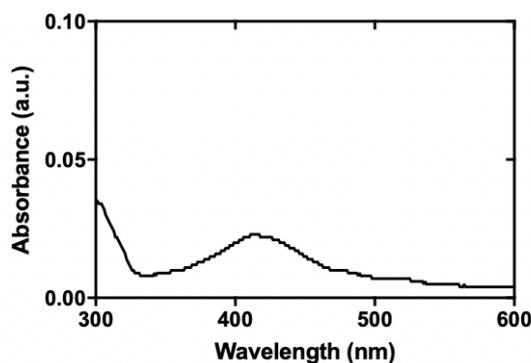


Figure 5 UV-Vis spectrum of AgNPs released from Ag-coated hydrogels.

Conclusions

In this study, the semi-direct contact test with transwell inserts provided a relatively quick approach and reliable results comparable to the conventional extract test in evaluating the cytotoxicity of hydrogel sheet dressings. For the Ag-coated hydrogel sheets, the localized effect of AgNPs released from the sample could be visually assessed as depleted formazan area by MTT assay in the semi-direct contact setup as opposed to the global effect obtained from the extract test. This semi-direct contact test with transwell inserts can be applicable to the routine evaluation of potential toxicity from medical devices.

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