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In Vitro Evaluation of PEG Modified Polyurethanes in Cellular Toxicity

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In vitro cytotoxicity of polyurethane(PU) and modified PUs (PU-PEG1K-OH, PU-PEG1K-NH_2, PU-PEG1K-SO_3, and PU-PPG1K-OH) was investigated by a direct contact and an extract dilution method. High density polyethylene was used as a negative control and PU containing 0.1% zinc diethyldithiocarbamate, as positive control. When the specimens was contacted directly with the cultured L929 cells, the positive control demonstrated significant toxicity (zone index=3), while all the other materials were non-toxic (zone index=0). These results indicated that all the specimens are not cytotoxic through short term physiochemical interaction toward the cultured cells. Basesd on an extract dilution method, viable cell numbers of the cultured L929 cells in each diluted extract were observed. The positive control demonstrated a significantly reduced cell viability % (IC_{50} % = 5.35). While, all the specimens demonstrated significant cell growth differences against the positive control ($p<0.001$). In conclusion, surface modified PUs seem to be non-toxic through either direct contact or extract dilution cytotoxicity testing method.

Key words : Polyurethane, Sulfonated PEG, Cytotoxicity, L929

INTRODUCTION

Polyurethane (PU) elastomer has been widely used for a variety of biomedical applications, especially as a raw material for the blood-contacting devices due to its suitable mechanical and non-thrombogenic properties.[1,2] It has been reported that a novel surface modification using sulfonated polyethylene oxide graft on PU (PU-PEG-SO_3) has improved blood compatibility, biostability and bacterial/calcification resistances in *in vitro, ex vivo* and *in vivo* tests. It is assumed as a synergistic effect of the highly hydrated PEG chain and negatively charged sulfonate (SO_3) groups.[3-7]

The physical, chemical and mechanical chracteristics of PU or surface modified PU has been well defined, but the biological properties of them has been investigated insufficiently. Cytotoxicity test, as an fundamental tool for toxicities of the biomaterials, has been suggested as an useful method for ensuring biological safety.[2,8] Appropriate toxicity testing protocols always include mammalian cell culture.[9-13] Three primary cell culture assays has been used for cytocompatibility evaluation: direct contact, agar diffusion, and elution (also known as extract dilution).[14-18] These three assays differ in the manner by which the test material is exposed to the cell. As indicated by the nomenclature, the test material may be placed directly on the cells in direct contact test, indirectly on the agar in agar diffusion test, or extracted in an appropriate solution that is subsequently placed on the cells in extract dilution test. The extract dilution test and agar diffusion test are useful to evaluate cellular toxicity of leaching substances from materials or devices. The direct contact test provides informations about the effects on cell-material interfaces as well as the toxicity of leaching substances.[2]

The aim of this study was to evaluate the cytotoxicity of PU and surface modified PU using both the direct contact and extract dilution test. The surface modified PUs include the surface grafted PUs using polyethylene glycol (PEG) chains (M.W. 1000) with pendent groups of -OH, -NH_2, -SO_3 (PU-PEG1K-OH, PU-PEG1K-NH_2 and PU-PEG1K-SO_3, respectively), the surface grafted PU with polypropylene glycol (PPG) chain (M.W. 1,000, PU-PPG1K-OH).

MATERIALS AND METHODS

Preparation of PU film

Polyurethane films were cast from polyurethane

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(Pellethane®, Dow Chemical Co., Midland, MI) by solvent film casting. These were extracted with methanol for 3 days using soxhlet apparatus and dried at 40°C under vacuum. Sulfonated PEG was synthesized using diaminoterminated-PEG (Nippon Oil and Fats Co., Tokyo, Japan) and propane sulfone (Sigma Chemical Co., St. Luise USA) as described previously.[5] Polyurethane surfaces were modified by grafting PEG chains with different end group (-OH, $-NH_2$ and $-SO_3$ respectively) and PPG chains (PU-PEG-OH, PU-PEG-NH_2, PU-PEG-SO_3 and PU-PPG-OH), as previously reported in details elsewhere.[4-7] Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co.

Specimen preparation

All the test materials was sterilized using ethylene oxide gas before cytotoxicity test. Two reference controls were purchased from Food and Drug Safety Center in Japan and used for cytotoxic test. High density polyethylene (HDPE) was used as the negative control and PU containing 0.1% zinc diethyldithiocarbamate (ZDEC) as the positive control. To evaluate the cytotoxicity of possible leachables from the test materials, test materials (1×5 cm) were immersed in Eagle's minimum essential medium (Gibco BRL, NY, USA) supplemented with 5% fetal bovine serum (Cansera, Canada) and penicillin G : streptomycin (10,000 units/ml : 10,000 μg/ml, Gibco Co. USA) (FBS-Eagle's MEM), for 72 hours at 37°C.[19] The ratio of the surface area of specimen to the culture medium was 3 cm^2/ml and these culture media were then used for extract dilution test.

Cell culture

L929 (fibroblastic mouse subcutaneous, ATCC CCL1) were obtained from the Korean Cell Line Bank and grown in 5% FBS-Eagle's MEM at 37°C in a humidified atmosphere of 5% CO_2 in air. At the time of the cytotoxicity test, the cells were dissociated with trypsin-ethylenediaminetetraacetic acid solution (Gibco BRL, NY, USA) and resuspended in 5% FBS-Eagle's MEM.

In vitro cytotoxicity : Direct contact method

The fibroblasts (4×10^5 cells/well in FBS-Eagle's MEM) were evenly seeded into 6 well plates (35 mm in diameter), in 3~6 replicates. The cultures were incubated for 2 days in a 5% CO_2 incubator at 37°C. After 2 days, the medium was replaced with 5% FBS-Eagle's MEM. Test specimen (1×1 cm) was placed on the centre of the medium and incubated for 24 hours. The specimens were removed after 24 hours incubation and the well plates were washed with phosphate buffered saline (PBS) and stained by 0.2% crystal violet (CV, Gibco BRL, NY, USA)-ethanol solution for 20 minutes. Normal cells in unaffected areas stained well in crystal violet, however, dead cells were detached from well plate during the staining process. The affected area below the specimens was detached and transparent and could therefore be easily discriminated from the unaffected and sufficiently stained area. The results were compared with two reference controls and interpreted by the value of zone index (Table 1).

Table 1. Zone description

Zone Index	Description of Zone
0	No detectable zone around or under specimen
1	Zone limited to area under specimen
2	Zone extends less than 0.5 cm beyond specimen
3	Zone extends 0.5~1.0 cm beyond specimen
4	Zone extends greater than 1.0 cm beyond specimen but does not involve entire dish
5	Zone involves entire dish

From ASTM.[16]

In vitro cytotoxicity : Extract dilution method

The CV staining method was performed as described by P.A. Holobaugh *et al.*, and H. Itagaji.[20,21] with some modification. The extracts of specimens were prepared by shaking for 72 hours at 37°C and diluted by adding the culture medium including serum (100%, 50%, 25%, 12.5%, 6.3%, 3.2%). Cell suspensions (2×10^4 cells/well) were seeded into each well of a 24 well plate in 3~5 replicates and incubated at 37°C for 24 hours in order to attach them to the bottom of the well. The medium was replaced by the extract or its dilution and incubated for 24 hours. The plate was then washed in PBS and stained by CV-ethanol solution and transferred into 96 well plate. Absorbance of each well, was measured at 610 nm by an automatic microplate reader. The absorbance of the blank wells, which contained no material, was regarded as 100% and the percentage absorbance for each well was calculated. The IC_{50}(%), i.e., the dilution % of extract out of test material inhibiting growth of cells by 50%, was estimated from the relative cell viability %. The relative cell viability is expressed as percentage of the optical densities in the medium containing with diluted extracts to the optical densities in the fresh control medium. The results of the experiments were investigated statistically, compared with the control, using t test.

RESULTS AND DISCUSSION

The cytotoxic test was performed with PEG/PPG modified materials (PU, PU-PEG1K-OH, PU-PEG1K-NH_2,

Table 2. The numerical values for Zone Index of the materials

Materials	Formed Zone Size (cm)	Zone Index
Negative control	-	0
Positive control	0.79±0.08	3
Polyurethane(PU)	-	0
PU-PEG1K-OH	-	0
PU-PEG1K-NH_2	-	0
PU-PEG1K-SO_3	-	0
PU-PPG1K-OH	-	0

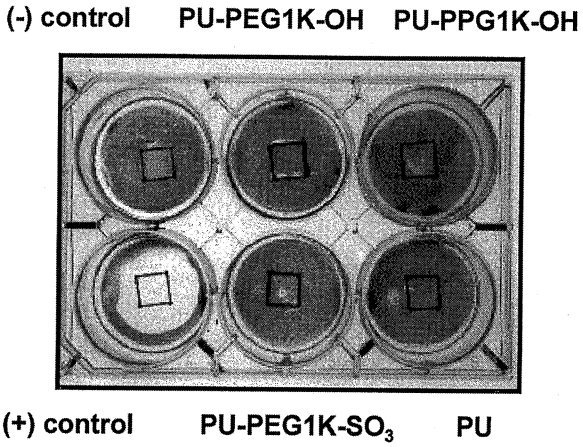

Figure 1. Cytotoxicity for materials by direct contact method using L929 cell monolayer.

PU-PEG1K-SO_3 and PU-PPG1K-OH). We examined the toxicity of leachables from test materials using the extract dilution and direct contact test. As a result of direct contact between cells and the specimens, the extents of cell proliferation are summarized in Table 2 and Figure 1. Figure 1 shows that the positive control had the distinct zone around of specimen, which was produced when toxic specimens promote neither cell proliferation, nor cell adhesion. Whereas, negative control or all the other specimens did not produce the zone, suggesting that specimens may be non-toxic, encourage cells to grow in a single layer and good cellular attachment. In addition, direct contact test can also provide the supplementary information for the effects on cell-material interface as well as the toxicity of leaching substances. Results from negative/positive control and test materials are shown in this Figure 2. The cells under and around positive control was detached on the culture dish and appeared round-shape. In the case of all modified PUs and PU, however, the cells appeared to be well attached and did not show the change of morphology, compared with negative control. Generally, direct contact test have various advantages : 1) mimic the physiological condition, 2) zone of diffusion (a concentration gradient of toxic chemical), 3) eliminate extraction preparation. But the major difficulty with this assay is the risk of physical trauma to the cells

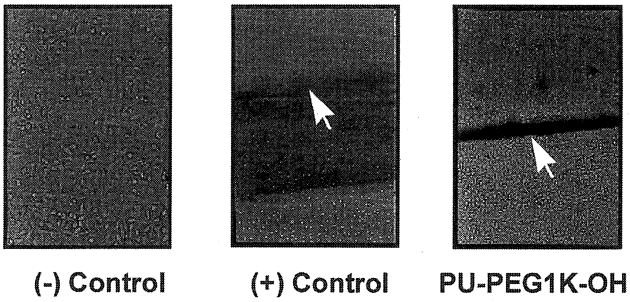

Figure 2. The morphology of cultured L929 cells under and around materials (*Arrow indicates the materials).

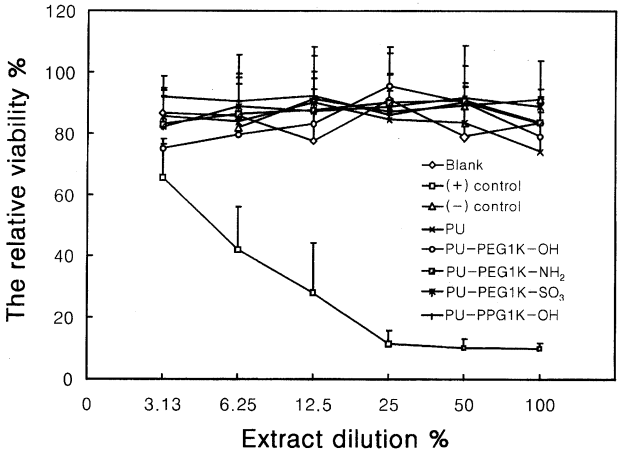

Figure 3. The relative viability % for L929 cell according to the dilution % of extracts.

from either movement of the sample or crushing by the weight of a high density sample.[22] The cells under specimen shown in Figure 2 demonstrate that the damage such as physical trauma is not exist.

The characteristics of the cells cultured in the presence of the extracts from the various test materials are summarized in Figure 3. Viable cell numbers of the cultured cells in each diluted extracts was observed. In the case of positive control, the value of IC_{50}(%) is about 5.35 ($y = 10.40+89.00\ e^{-x/6.6}$, $r^2 = 0.9961$). However, all the other specimens was not measured the value of IC_{50}(%) and was the significant difference of cell growth against the positive control ($p<0.001$). These results show that PEG modified PUs is nontoxic. *In vitro* methods for cytotoxicity should quantify cell viability and growth and be correlated in vivo methods. The correlation with animal tests will depend on the nature of the extraction solvent. Although extract dilution test provides a quantitative comparison with positive/negative control, it still have the difficulty in the preparation of extract. In our experiments, culture medium containing serum was used as extraction solvent and test materials extracted at 37°C. When culture medium containing serum was used as extract solvent, both polar and nonpolar components could be extract-

ed.[22] Also, the use of culture medium containing serum had the higher toxicity than saline.[23] It is reported that in many case toxicity of polymeric biomaterials, if any, seems to be due to their leachables.[24] Therefore, surface modifying process used in this study is thought to be adaptable for cytocompatible biomaterial.

CONCLUSION

Surface modified PUs have shown to be non-cytotoxic, evaluated by either direct contact or extract dilution method. Considering the results of the direct contact and extract, tested specimens may not have a serious influences on cells, when they are applied to the tissue. We have previously reported that sulfonated PEG grafted PU has improved blood compatibility, calcification-resistance and infection-resistance *in vitro, ex vivo* and *in vivo* as compared to other test materials. This study suggest that sulfonated PEG grafted PU is cytocompatible in addition to antithrombogenic, calcification-resistant and infection-resistant.

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