Polymer(Korea), Vol. 40, No. 2, pp. 225-231 (2016)
http://dx.doi.org/10.7317/pk.2016.40.2.225

ISSN 0379-153X(Print)
ISSN 2234-8077(Online)

생체재료로서 적용을 위한 폴리케톤의 생물학적 안전성 평가

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(2015년 9월 25일 접수, 2015년 10월 28일 수정, 2015년 11월 10일 채택)

Biological Safety Evaluation of Polyketones as Biomaterials

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(Received September 25, 2015; Revised October 28, 2015; Accepted November 10, 2015)

초록: 생체재료는 의료기기로서 임상에 적용되기 위해서는 생물학적 안전성 평가에 의한 생체적합성 확보가 필수적이다. 이번 연구에서는 폴리케톤 고분자의 생체재료로서 사용 가능성을 평가하기 위해 생물학적 안전성 평가를 실시하였다. 폴리케톤 고분자의 용출물로 고분자의 생체적합성을 판단하는 생물학적 안전성 초기실험인 세포독성, 유전독성, 피부자극성, 감작성, 전신독성 시험을 실행하였다. 모든 시험에서 폴리케톤 고분자는 독성을 유발하지 않음을 확인할 수 있었으며, 이러한 결과는 폴리케톤 고분자가 생체재료로서 생물학적 안전성이 있으며 비분해성 고분자를 대체할 수 있을 것으로 판단된다.

Abstract: Biomaterials for clinical applications such as medical and implant devices require evaluation to determine their biocompatibility. This study aimed to evaluate the initial biological safety of polyketone (PK) polymers, which have potential use as biomaterials. It presents results from cytotoxicity and genotoxicity assays and tests determining the levels of skin irritation, sensitization, and acute systemic toxicity caused by PK. The PK polymers showed no cytotoxicity, genotoxicity, allergenicity, irritancy, and systemic toxicity. These results indicate that the PK polymers are potentially biological safe and could be used to replace non-degradable polymeric biomaterials.

Keywords: polyketones, biomaterials, biocompatibility, biological safety.

Introduction

Synthetic polymers have played an important role in biomedical applications such as in the modulation of wound healing, as implantable medical devices, artificial organs, and prostheses. They have also been used in ophthalmology, dentistry, bone repair, and drug delivery systems.[1,2] The term biomaterials is used to describe materials that can interact with biological systems to evaluate, treat, augment, or replace any tissue, organ, or body function.[3-5] Polymeric biomaterials are relatively easy to manufacture into products with various shapes and desirable mechanical and physical properties, at a reasonable cost. However, one of the major limitations of these materials is their biocompatibility.[3] The most important requirement for polymeric biomaterials is biocompatibility, which describes the ability of a material to perform with an appropriate host response in a specific application.[6] Biocompatibility consists of biological safety and biofunctionality. Biological safety requires appropriate systemic and local (surrounding tissue) host responses, the absence of cytotoxicity, mutagenicity, and carcinogenicity. These characteristics determine the suitability of a material with respect to its potential

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detrimental effects on the body (toxicity), and potential detrimental or beneficial effects of the physiological environment on the performance of the material. Biofunctionality refers to the ability of a material to perform the intended task.[7,8]

Aliphatic polyketones (PKs) are a family of polymers produced by the polymerization of olefin and carbon monoxide (CO) copolymers using ethylene or propylene or both, with CO as a co-monomer.[9,10] PK could be considered high-performance thermoplastic polymers that combine the features of good mechanical properties, chemical resistance, low gas permeability, and durability. PK material is cheaper than general engineering plastics such as polyamide, polyester, and polycarbonate.[11,12] In addition, there is great interest in developing environmentally friendly materials to replace the conventional engineering plastics.[13] However, there have been no reported biomedical applications of these biomaterials, despite their several advantages.

In the present study, the initial biological safety of the PK polymer was evaluated, as a potential means of determining its biocompatibility, using cytotoxicity, allergenicity, sensitivity, genotoxicity, and acute toxicity tests. The studies were carried out in compliance with the rules of the International Standards Organization (ISO 10993/EN 30993, biological evaluation of medical devices).

Experimental

Extraction Conditions for PK. The PK was kindly provided by Hyosung (Seoul, Korea). The glass transition and melting temperatures were 10 and 225 ºC, respectively while the melt index was 60 g/10 min. The PK used in this study was an alternating terpolymer prepared using ethylene, propylene, and CO as monomers. The PK pellets were sterilized by ethylene oxide gas, chopped, and extracted in desired solutions at a proportion of 0.2 g/mL at 37 ºC for 72 h, according to the ISO standard methods for preparing samples.[14]

***In Vivo* Experiment.** All animal experiments, and treatments were performed in accordance with the guidelines of the animal experiment and ethics committee of Yonsei university college of medicine. Protocols were reviewed and approved by the institutional animal care and use committee (IACUC) of the Yonsei laboratory animal research center (YLARC).

***In vitro* Cytotoxicity Test.** The L-929 cell line (American Type Culture Collection, ATCC, Manassas, VA, USA) was cultured in Minimal Eagle's medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco) at 37 ºC with 5% CO_2 in a humid environment. The cytotoxicity test was performed using the extract dilution method.[15] The L-929 cells were seeded in 6-well plates at a density of 5×10^5 per well and grown to a subconfluent monolayer. The extract was dissolved in a polar solvent and then serially diluted 2-fold with fresh medium containing 10% FBS. The cells were treated with the diluted extract medium and then incubated further for 24 h. Following the incubation, cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay (Amresco, Solon, OH, USA). The cells were incubated with MTT for the last 4 h of the culture period at 37 ºC in the dark. The medium was decanted, and the formazan salts produced were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). The absorbance of the final solution was determined at 570 nm using an automatic microplate reader (SpectraMax 340, Molecular Devices Co., Sunnyvale, CA, USA). The experiments were performed in triplicate. The negative and positive controls used were international standard reference materials including high-density polyethylene (HDPE) films (thickness 0.5 mm) and polyurethane (PU) films containing 0.1% zinc diethyldithiocarbamate (ZDEC, 0.5 mm thickness), respectively. The reference material was also extracted using the same method specified in the ISO guideline.[14]

Genotoxicity Test. Ames Test: The Ames test was performed using the S9 fraction (activation mix, composed mainly of the microsomal fraction from an Aroclor 1254-induced rat liver homogenate) as the metabolic activation system, as described previously.[16-18] Four strains of *Salmonella typhimurium* TA97, TA98, TA100, and TA1535 and one of *Escherichia coli* WP2uvrA were used in this experiment. The bacterial strains were cultured in tryptic soy broth (BD, San Jose, CA, USA) at 37 ºC for 18 h. The PK was extracted with 0.9% sodium chloride (saline, polar solvent) or DMSO (nonpolar solvent) at a proportion of 0.2 g/mL at 37 ºC for 72 h. The vehicle was prepared using the same extraction conditions for use as a negative control. The S9 contained enzymes, which perform several metabolic conversions similar to those of mammalian organs, were used in the test system for the detection of mutagenic chemicals. The mixture was composed of 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 8 mM magnesium chloride ($MgCl_2$), 33 mM potassium chloride (KCl), 5 mM glucose-6-phosphate, and 100 mM sodium phosphate buffer (pH 7.4). Briefly, the extracted samples, as well as the positive and negative control solutions,

were inoculated into the bacterial strains with and without the Aroclor-1254-induced rat liver fraction (S9, MolTox™, Boone, NC, USA). The components were sequentially added to 2 mL of warm, soft agar. The mixture was poured into a petri dish containing Vogel-Bonner minimal medium (1.5% agar in Vogel-Bonner E medium with 20 mg/mL glucose). After the soft agar had solidified, the petri dish was incubated at 37 ºC for 48 h. Then, the reverting colonies (His+) were counted. The experiments were performed in triplicate.

Sister Chromatid Exchange (SCE) Test: The sister chromatid exchange (SCE) test were performed in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines.[19] Chinese hamster ovary cells (CHO-K1, ATCC) were cultured in Dulbecco's Modified Eagles Medium (DMEM)/Nutrient Mix F12 (1:1, with glutamine) containing 10% FBS with 1% penicillin-streptomycin. Cells were grown to a subconfluent monolayer and then the medium was replaced with the diluted extract medium with or without the S9 fraction. Approximately 6 h later, the cell medium was replaced with fresh medium containing 10 μM 5-bromo-2-deoxyuridine (BrdU, Sigma) and the cells were incubated in the dark for 24 h. Then, the metaphases were blocked during the last 2 h of the incubation with 100 μM colcemid (Sigma). The cells were collected by centrifugation, incubated in 75 mM KCl for 20 min at 37 ºC, and then fixed in a methanol:glacial acetic acid fixative (3:1 v/v). The fixed cells were mounted on glass slides and air-dried overnight. Dry slides were stained using the modified Hoechst 33258 fluorescence-plus-Giemsa technique. The number of SCEs in 100 second-metaphase cells was counted from each of the cultures on coded slides using a microscope (×1000 magnification, Olympus, Japan). Methylmethanesulfonate (MMS) and cyclophosphamide (CPA) were used as positive controls for a direct acting compound and a compound requiring metabolic activation, respectively.

Micronucleus (MN) Tests: Micronucleus (MN) tests were performed in accordance with the OECD guidelines.[20] The sample was extracted with saline as the polar solvent and cotton oil (CSO, Sigma) as the nonpolar solvent. The extract samples were administered to mice by intraperitoneal injections. The CPA solution (2 mg/mL) was used as a positive control while normal saline was the negative control. A group of five mice was used for each sample. After a 48-h treatment period, the animals were euthanized, and the bone marrow cells in the femurs were collected in 0.5 mL FBS by centrifugation. The supernatants were removed, and the cell pellets were resuspended in remaining serum. The cell suspension was spread on a clean glass slide and air-dried. Following fixation in methanol for 20 min, the cells were stained with Giemsa solution (Sigma) and the erythrocytes were counted under a microscope (Olympus, Japan). A total of 2000 polychromatic erythrocytes (PCEs) were scored per animal to determine the MN frequencies and 200 erythrocytes were examined to calculate the ratio of PCEs to normochromatic erythrocytes (NCEs).

Irritation Test. The evaluation of the irritancy level was performed using an intradermal reactivity test with 3-month-old male New Zealand white rabbits in accordance with the ISO guidelines.[21] The PK extract was dissolved in saline and CSO. A total of 0.2 mL of the extract dissolved in the polar solvent was injected intracutaneously, at five sites on one side of each rabbit. Similarly, 0.2 mL of the polar solvent (control) was injected at five posterior sites on the same side of each rabbit. The irritancy was determined by adding the average erythema and edema scores at 24, 48, and 72 h intervals and dividing this by the number of evaluation intervals. The irritation reaction was classified on the basis of a 0 to 4 score level according to the descriptive scale.[21]

Sensitization Test. The sensitization was determined using a guinea pig maximization test (GPMT) in accordance with the ISO guidelines.[21] The PK extract was prepared in saline and CSO. Equal volumes of the extract and Freund's complete adjuvant (FCA, Sigma) were combined and homogenized by continuous and vigorous vortexing until an emulsion was formed. For the intradermal induction phase, healthy young adult guinea pigs (n=5) weighing 300-500 g were injected with the extract (saline or CSO) and FCA mixture (saline or CSO extracts). Five guinea pigs each (negative controls using saline and CSO) were injected with the corresponding control blank and the FCA mixture. Five guinea pigs were injected with 2, 4-dinitrochlorobenzene and the FCA mixture as the positive control. On day 6, the injection sites were treated with 10% sodium dodecyl sulfate (SDS), and the next day the control and test animals were topically patched with the appropriate control blanks and test extracts, respectively. The patches were removed after a 48 h exposure. Following a 2 week rest period, the control and test animals were topically patched on a previously untreated area with the appropriate control blanks and test extracts, respectively. The patches were removed after a 24 h exposure. The dermal patch sites were observed for erythema and edema 24 and 48 h after patch removal. Each animal was assessed for a sensitization response based upon the dermal scores of the Magnusson and Kligman scale.[21]

Acute Systemic Toxicology Test. An acute systemic toxicology test was conducted based on the procedures described in the ISO guidelines.[22] The sample was extracted with saline (polar solvent) and CSO (nonpolar solvent). The test animals used were ICR mice weighing 17-23 g. Each extract was intravenously injected into five test animals, and the vehicle (negative controls) was similarly injected into five control animals. The animals were observed immediately after and 4, 24, 48, and 72 h after the injection. All animals were weighed prior to the injection and after the final observation.

Statistical Analysis. All the quantitative data shown represent the mean ± standard deviation (SD). Statistical analyses were performed by an analysis of variance (ANOVA) using the statistics for the social sciences (SPSS) software. The differences were considered statically significant at $p<0.05$.

Results

Cytotoxicity Study. The *in vitro* cytotoxicity was evaluated using the extract dilution method by incubating cells with the extract of the PK polymers. The cytotoxicity test is useful for assessing toxicity using a mammalian cell culture system, and has been adopted for primary safety screening of biomaterials prior to *in vivo* testing.[23] The cell viability was determined using the MTT assay. A decrease in cell viability is indicated by a decrease in the metabolic activity of the cells. The viability of cells treated with different concentrations of the PK polymer did not differ significantly from that of the negative control cells (Figure 1). In addition, we did not observe any changes in cell morphology or cellular lysis on the cell monolayer (data not shown). The ISO 10993-5 has reported that the reduction of cell viability to < 70% of the blank, indicates cytotoxic potential.[15] The extract at a concentration of 50% of the test sample should have a viability that is at least the same or higher than that of the extract at 100%. This result clearly indicates that the PK polymer extracts are not toxic.

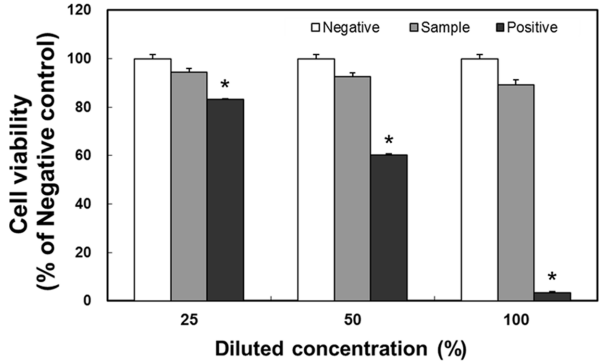


Figure 1. Results of the cytotoxicity test. Mean values of cell viability obtained using the MTT assay in the L-929 cell line (n=3). Results are expressed as means±SD. *$p<0.001$ compared to the negative control of each group on diluted concentration, ANOVA.

Genotoxicological Analysis. Genotoxicity includes all potential mutagenic toxicities with serious consequences resulting in genetic damage. The genotoxicity tests use mam-

Table 1. Results of the Ames Tests

Solvent		S9	Revertants/Plate (n=3)				
			Salmonella typhimurium				*Escherichia coli*
			TA97	TA98	TA100	TA1535	WP2uvrA
Nonpolar extract	Negative control	S9 (–)	405±12	487±38	317±4	32±6	141±19
		S9 (+)	407±46	335±55	48±3	21±15	239±81
	Sample	S9 (–)	389±37	405±12	302±13	35±12	132±4
		S9 (+)	595±55	351±29	45±4	21±6	207±62
Polar extract	Negative control	S9 (–)	383±34	422±12	291±15	32±9	138±3
		S9 (+)	409±70	379±76	46±4	27±2	244±20
	Sample	S9 (–)	389±12	370±60	302±13	34±7	142±9
		S9 (+)	384±36	344±77	38±6	20±9	235±54
Positive control		S9 (–)	>5000	1388±64	1004±25	611±119	1055±106
		S9 (+)	>3000	2196±628	1852±143	757±164	2201±666

Positive controls without S9 activation were ICR-191 for strain TA97, 4-nitroquinoline N-oxide for strain TA98, methyl methanesulfonate (MMS) for strain TA100, sodium azide for strain TA1535, and MMS for *E. coli* WP2uvrA. Positive controls with S9 activation were 2-aminoanthracene for strains TA97, TA98, TA100, and TA 1535 and MMS for *E. coli* WP2uvrA.

Table 2. Results of the Sister Chromatid Exchange (SCE) Tests

Group (n=3)	S9	Negative control	Sample (12.5%)	Sample (25%)	Sample (50%)	Positive control
The number of SCEs/ chromosome	S9 (−)	0.44±0.09	0.44±0.10	0.45±0.08	0.45±0.10	2.07±0.67*
	S9 (+)	0.44±0.10	0.45±0.10	0.44±0.10	0.44±0.10	1.50±0.37*

*p<0.001 compared to the negative control, ANOVA.

Table 3. Results of the Micronucleus (MN) Assay

Solvent	Group (n=5)	MN/PCE (%)	PCE[a]/NCE[b] ratio
Nonpolar extract	Negative	1.78±0.25	9.40±1.01
	Sample	1.74±0.15	7.23±0.97
Polar extract	Negative	1.74±0.12	5.92±2.61
	Sample	1.64±0.12	6.86±1.72
Positive control		10.12±0.57*	0.59±0.05*

[a]PCE, polychromatic erythrocyte. [b]NCE, normochromatic erythrocyte. *p<0.05 compared to the negative control, ANOVA.

malian or non-mammalian cells to evaluate whether the test materials cause gene mutations, chromosomal aberrations, or other DNA or gene changes. In these tests, *in vitro* and *in vivo* assays are usually used to assess substances or materials that can directly or indirectly induce genetic damage through a variety of mechanisms.[6,18]

As shown Table 1, the average number of revertant colonies of each tester strain treated with nonpolar or polar solvent PK extracts did not differ significantly from that of the negative control. Therefore, there was no induction of revertant colonies with or without metabolic activation. In contrast, the positive control cells showed typical mutagenic toxicity as suggested by the induction of revertant colonies in all the five tester strains in the presence and absence of metabolic activation. The results demonstrate that the PK polymer is not mutagenic as determined by the bacterial reverse mutation assay.

The mutagenicity of the PK polymer was determined using the SCE method in CHO-K1 cells in the presence or absence of the metabolic activation system. The frequencies of SCE in the chromosome of mammalian cells in metaphase are shown in Table 2. The SCE was observed in the negative control group but not the PK extract-treated group with or without the metabolic activation (S9 mix). In addition, the positive control (p<0.001) but not the PK extracts caused concentration-dependent increases in the frequencies of the SCE.

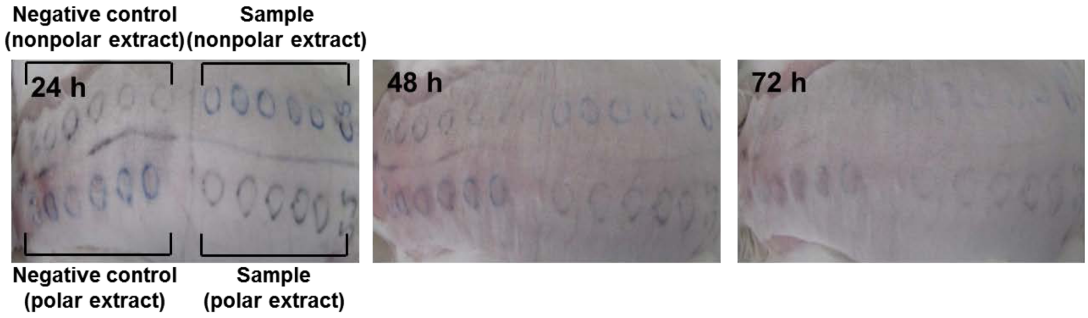
A MN test was performed to evaluate the mutagenic potential of the PK polymers, using 8-week-old male ICR mice. The PK extracts dissolved in nonpolar, and polar solvents did not induce any significant increase in the percentage of MN per PCE. In addition, there was no significant decrease in the PCE/NCE rate, which is an indicator of the genotoxicity of sample-treated groups compared to the negative control. In contrast, the positive control significantly increased the MN and decreased the PCE/NCE ratio (Table 3). From these results, it can be concluded that the PK polymer did not show any mutagenic potential under conditions of the mammalian erythrocyte MN test.

Intra-cutaneous Reactivity Study. The irritation test evaluates a localized irritation potential that causes a localized inflammatory response following exposure to a biomaterial.[6] In this study, irritation was determined in rabbits using an intradermal injection. As shown Figure 2(A), the negative control, and PK extract were each injected into five sites of three animals, and the erythema and edema were observed at 24, 48, and 72 h after the injection. Erythema or edema was not found in any of the test animals at any time in both the negative control and the saline PK extract-treated groups. However, it was discovered that animals showed erythema and edema after the intradermal injection of the nonpolar CSO extract (Figure 2(B)). The average irritation score was 0.99 and 0.52 in the negative control and the group treated with the PK sample extracted with the CSO, respectively. A grade in the range of 0.5-0.9 implies a very slight and barely perceptible irritation.[21] Therefore, the grade of the PK extract-treated group was lower than that of the CSO-treated group. The irritation potential induced by the samples was comparable to that of the control. The results indicate that the PK polymers do not induce potential irritation of the skin.

Sensitization Study. The potential of the PK polymers to produce allergic responses was evaluated in the GPMT. The challenge patches were removed after 24 and 48 h, and no erythema or edema formation was observed at the test sites of the animals induced and challenged with the PK extracts. For the positive control group, all the animals showed a grade 2 skin reaction at all reading time points after the challenge (Table 4).

Acute Systemic Toxicity Study. The systemic toxicity test evaluates the potential harmful effects on target and organs fol-

A



B

Group	24 h		48 h		72 h		Average score
	Erythema	Edema	Erythema	Edema	Erythema	Edema	
Negative control (polar extract)	0	0	0	0	0	0	0
Negative control (nonpolar extract)	0	0	0.9	1.1	1.7	2.3	0.99
Sample (polar extract)	0	0	0	0	0	0	0
Sample (nonpolar extract)	0	0.3	0.5	0.7	0.6	1.1	0.52

Figure 2. Results of the intradermal reactivity analysis in the skin irritation test (n=3). (A) Macroscopic; (B) Cumulative grading evaluations.

Table 4. Results of the Guinea Pig Maximization Test (GPMT) in the Skin Sensitization Test

	Negative control (nonpolar extract)	Sample (nonpolar extract)	Negative control (polar extract)	Sample (polar extract)	Positive control
24 h	0	0	0	0	2
48 h	0	0	0	0	2

Number means the average grade by the Magnusson and Kligman scale.

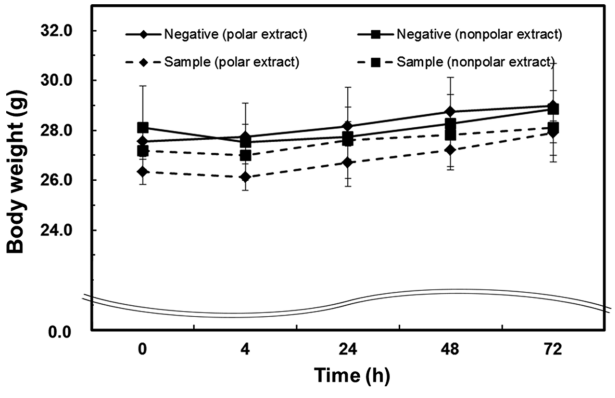


Figure 3. Changes in body weight of mice in the acute systemic toxicity test (n=5).

lowing single or multiple exposures to biomaterials.[6] In this study, the ICR mice were intravenously administered the PK extract prepared with the polar or nonpolar solvent. Abnormal signs were not observed in the general appearance and body weight of the mice after the injections (Figure 3). The body weight of both the negative control and sample-treated groups increased 72 h after the injection. All animals appeared to be active and healthy during the study period (data not shown). This finding suggests that the PK polymer did not induce any acute toxicity after the single intravenous injection.

Discussion and Conclusions

It has been reported that PKs possess excellent properties like rapid crystallization, high tensile strength, high chemical and wear resistance, very low permeability, and good impact behavior over a broad temperature range.[24-26] These properties make the PKs suitable materials for engineering applications and they could be possible replacements for polymers like polypropylene or polyethylene in some specialized areas of

drug delivery, bioengineering, optical devices, and other applications.[27-30]

Biocompatibility evaluation of a material is an essential step for its acceptance as a biomaterial, in addition to the testing of its physical properties. Biocompatibility studies require complex *in vitro* and *in vivo* experiments. *In vitro* cell culture studies are usually the first step in the evaluation of the biocompatibility. *In vivo* biological reactivity tests are designed to determine the biological responses of animals to the materials following direct or indirect contact or injection of specific extracts prepared from the material.[8,31,32]

We examined the feasibility of using PK polymers as biomaterials by assessing their cytotoxicity, mutagenicity, irritancy, sensitivity, and acute systemic toxicity. The present study focused on the initial biological safety of the PK polymers and has revealed that they had a good level of safety under the chosen test condition. Additional studies involving subchronic and reproductive toxicities as well as implantation tests to estimate the biological reactivity of the polymers should be performed. This would enable the assessment of the final suitability of the candidate medical device formulated with these polymers for human applications.

In conclusion, the PK polymers may be regarded as biomaterials with the potential to demonstrate good biocompatibility for some clinical applications.

Acknowledgments: This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health & Welfare, Republic of Korea (A120878).

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