Cytotoxicity study of homopolymers and copolymers of 2-hydroxyethyl methacrylate and some alkyl acrylates for potential use as temporary skin substitutes

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The cytotoxicities of both homopolymers and copolymers of 2-hydroxyethyl methacrylate (HEMA) and various alkyl acrylates (AA) were investigated using cell culture techniques. The particular alkyl acrylates used were methyl acrylate (MA), ethyl acrylate (EA) and butyl acrylate (BA). The AA contents of the polymers studied were in the range of 0–25% by weight. They were synthesized via bulk polymerization in the form of thin sheets of 0.5 ± 0.1 mm thickness. After sterilization using ethylene oxide gas, cytotoxicity tests, including both direct and indirect contact tests, were performed using cell lines of L929 and normal human dermal fibroblasts. The results showed that P(HEMA-co-EA) with EA contents of 15–25% and P(HEMA-co-BA) with BA contents of 10–25% appeared to be non-cytotoxic and attractive for more specific cytocompatibility tests and *in vivo* studies with a view to their use as temporary skin substitutes.
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1. Introduction

Poly(2-hydroxyethyl methacrylate), PHEMA, is a well-known hydrogel, widely used in soft contact lenses [1]. In addition, PHEMA hydrogels have also found use in various other applications, such as drug release systems and wound dressings [2–4]. Since PHEMA does not allow cell adhesion and growth on its surface, its biomedical applications are limited to those in which cell cohesion is detrimental. Consequently, modifications of PHEMA have been performed by several means, for instance, PHEMA–gelatin interpenetrating polymer networks (PHEMA–gelatin IPN) [5], phosphorylated PHEMA sponges and gels [6], and HEMA-methyl methacrylate capsules [7]. Sequential homo-interpenetrating polymer network sponges were also developed to improve the inherently poor mechanical strength of PHEMA sponges for uses as implantable devices [8]. *In vivo* biological responses to a tissue-integrating PHEMA sponge were investigated by Vijayasekaran *et al.* [9].

In the present study, the three alkyl acrylates – methyl acrylate (MA), ethyl acrylate (EA) and butyl acrylate (BA) – were copolymerized with HEMA in order to try to improve the balance of mechanical and water transport properties of thin sheets for potential use as temporary skin substitutes. The cytotoxicities of PHEMA and its copolymers were both qualitatively and quantitatively evaluated. Qualitative evaluation was based on morphological examination of cells in direct contact with the samples. Quantitative evaluation was performed via a modified elution test method in which the presence or release of unreacted monomers or degradation products leaching from the specimens was determined. The results give some indication of their biocompatibility and therefore their potential as biomaterials.

The attraction of PHEMA-based hydrogels for use as

temporary skin substitutes stems mainly from their well-established biocompatibility in soft contact lens applications and, in particular, their favorable water and oxygen transport properties. Not only does PHEMA readily absorb water up to an equilibrium water content of about 40% by weight, it also releases the absorbed water when left in contact with air at an appropriate rate. Its water vapor transmission rate through thin films generally falls conveniently in between the evaporative water loss rates from typical first and second-degree burns. This delicate balance between water absorption and water vapor transmission is vitally important in a temporary skin substitute in order for it to be able to maintain the wound surface in a moist state (i.e. not too wet but not too dry) which is conducive to wound healing.

Consequently, when HEMA is copolymerized with alkyl acrylates (AA) in order to improve the rather poor mechanical strength of PHEMA, the accompanying effects on biocompatibility and water transport properties also need to be carefully considered. While the decreasing effect on the latter is within acceptable limits, and indeed can be put to good use in controlling water transport, the effect on biocompatibility is less well documented. This paper therefore focuses its attention on the effect of HEMA–AA copolymer composition on cytotoxicity and, in doing so, provides some indication as to the potential suitability of these materials for use as temporary skin substitutes.

2. Materials and methods

2.1. Preparation of hydrogels

The hydrogels studied comprised four homopolymers, namely: PHEMA, PMA, PEA, PBA, and the three copolymers of P(HEMA-co-MA), P(HEMA-co-EA) and P(HEMA-co-BA). Each monomer copolymerized with HEMA ranged from 5 to 25% by weight in the initial comonomer feed.

2.1.1. Purification of materials

The monomers, HEMA, MA, EA and BA, and the ethylene glycol dimethacrylate crosslinking agent, EGDM, were each purified by first drying with anhydrous sodium sulfate and then distilling under vacuum. The constant boiling fractions were collected and stored in the refrigerator until required for use in polymerization. Benzoyl peroxide, the free radical initiator used in this work, was purified by recrystallization. Crude benzoyl peroxide was first dissolved in chloroform. After filtering off any insoluble impurities, methanol was added and the benzoyl peroxide was allowed to recrystallize in a refrigerator at 0–5 °C. The white, needle-like crystals obtained were dried in a vacuum oven to constant weight at 40 °C and stored in a refrigerator until required for use.

2.1.2. Mold design

The *in situ* polymerization mold used in this work for making thin sheets consisted of two glass plates, 150 × 150 × 5 mm, each lined on the inside with poly(ethylene terephthalate), (PET) film to permit ease of separation and sample removal. A thin wire covered with Teflon® tape functioned as a spacer (0.5 mm thickness) in between the PET films and enclosed the internal volume into which the polymerization mixture was injected. The two glass plates were held securely together by means of metal clips.

2.1.3. Polymerization

Copolymers of HEMA and each of the three alkyl acrylates were prepared by mixing HEMA and the alkyl acrylate together in various compositions ranging from 0 to 25 wt % of alkyl acrylate. To each of the mixtures was then added 0.1 mol % of benzoyl peroxide and 1.0 wt % of EGDM at low temperature (0–5 °C) until a homogeneous clear solution was obtained. After outgassing with nitrogen, the mixture was injected into the mold. The mold was then placed in an oven at 60 °C for 3 days followed by 2 h postcure at 110 °C under vacuum. Any unreacted monomers ($< 0.5\%$ by wt) were removed from the polymer films by soaking in distilled water for one week followed by 80% aqueous methanol for 2–3 days.

2.2. Cytotoxicity tests

Two types of cells were used in this assay: first, a cell line of L929, mouse connective tissue, fibroblast-like cells; and, second, normal human dermal fibroblasts (NHF) (passage 3–9). Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), together with penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Once 80% confluence was reached, the cells were subcultured for cytotoxicity study. The hydrogel samples were sterilized with ethylene oxide gas. Cutinova Thin, a commercial dressing was studied as a reference material.

2.2.1. Direct contact test

This assay conformed to ISO 10993-5 [10] and USP24 [11] with slight modifications. The hydrogel samples were cut into small circular discs of diameter 5 mm. L929 cells were seeded onto a 35 mm dish at a density of 2.2×10^4 cells/dish and incubated for 72 h. The growth medium was removed and a sample disc placed in the middle of the dish. 600 μl of fresh medium were then slowly added to each dish. Cell morphology and the toxic zone were evaluated after a 24-h exposure to the cells. The cells were stained with 0.01% neutral red in PBS for membrane integrity. High-density polyethylene (HDPE) and natural rubber containing carbon black were used as negative and positive controls respectively. The toxic zone of each sample was deduced from an average of eight readings around the specimen. Each sample was tested in duplicate and repeated whenever there was any uncertainty about the results obtained.

2.2.2. Indirect contact test

This assay was tested with L929 cells and normal human fibroblasts and was a modified version of conventional direct and indirect contact tests. The cells were seeded

onto a 96-well plate at a density of 10^3 cells/well and incubated for 24 h. A disc of each hydrogel was held in the growth medium above the cell monolayer in each well using a needle. Each sample was tested in 4 wells, while wells without specimens were used as controls. Cells were exposed to the hydrogel samples for 48 h. The specimens were removed from each well and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed as previously described [12]. Briefly, a stock solution of MTT was prepared at a concentration of 5 mg/ml in PBS and 50 μl of the MTT solution were added to each well of 200 μl growth medium. The plate was then incubated at 37 °C for 4 h. The medium and MTT were removed from the wells and the MTT-formazan crystals produced by the cells dissolved in 200 μl of DMSO in each well. The absorbance was measured at 570 nm using a microplate reader.

The choice of MTT in preference to other assays was based on its rapidity, precision and the absence of washing steps that would have otherwise increased processing time and sample variation. Ideally, a colorimetric assay for living cells should utilize a colorless substrate that is modified to a colored product by any living cells but not by dead cells or the culture medium. The MTT assay satisfies these requirements and has gained widespread use for this purpose.

2.3. Statistical analysis
Statistical analysis was performed using one-way ANOVA followed by Scheffe for multiple comparison with a 95% confidence interval (CI).

3. Results
3.1. Direct contact test
Cell morphology and cell membrane integrity were examined after a 24-h exposure to the hydrogel samples. Neutral red staining was useful for differentiating viable and non-viable cells, facilitating the determination of the toxic zone. Photographs of L929 cells adjacent to the negative and positive controls as well as some of the hydrogels tested are shown in Fig. 1(a)–(e). The toxic zone was also evaluated and the reactivity graded according to the guidelines given in USP24. The results are shown in Table I. PHEMA and P(HEMA-co-5%MA) showed mild to moderate cytotoxicity while the copolymers containing 10–25% MA exhibited mild toxicity. P(HEMA-co-5%EA) showed moderate toxicity, P(HEMA-co-10%EA) was mildly toxic to L929, but the copolymers with 15–25% EA and the homopolymer PEA were non-toxic. Whereas the P(HEMA-co-5%BA) exhibited moderate toxicity, the 10–25% BA compositions were non-toxic. A toxic zone was not found around Cutinova Thin. In this latter case, cell morphology under the sample could not be examined due to the opaqueness of the specimen.

3.2. Indirect contact test
The most widely used technique for this purpose involves the use of a tetrazolium salt, MTT. In this work, an MTT assay was performed to quantify the relative cell numbers, an approach based on the function of mitochondria in living cells to metabolize the MTT to a colored formazan salt. The assay was carried out after the cells had been exposed to the hydrogel samples for a period of 48 h. The absorbance was reported both as a mean value and as a 95% CI for the mean. The results are shown in Fig. 2(a) and (b) for the L929 cells and the normal human fibroblasts respectively. For the L929 cells, the results showed that some hydrogels significantly inhibited cell growth at 95% CI ($p < 0.05$) as indicated in Fig. 2(a). P(HEMA-co-5%EA) and P(HEMA-co-5%BA) showed the most severe cytotoxicity with 7–9% viability relative to controls. Cutinova Thin showed moderate cytotoxicity to the L929 cells, exhibiting 34% viability relative to controls. Some of the samples also inhibited growth of normal human fibroblasts significantly at a p-value < 0.05. P(HEMA-co-5%EA), P(HEMA-co-5%BA) and Cutinova Thin exhibited similar degrees of cytotoxicity, showing 20–24% viability relative to controls.

4. Discussion
PHEMA has previously been described as a poor cell attachment substrate but did not appear to show significant intrinsic surface toxicity (IST) [13]. However, the results from this study have shown that PHEMA inhibits cell growth of L929 cells and NHF showing 52–65% viability relative to controls. These results are similar to other previous reports [5, 14]. Horák *et al*. reported that concentrated extracts of PHEMA beads significantly reduced the proliferation activity of Hep2 cells after a 72-h exposure but that this inhibiting effect was not observed when the cells were exposed to the extracts for 24 h. Long-term, stationary extraction of the homo and copolymers studied in this present work was carried out at 37 °C for 10 days with a ratio of sample surface area to serum-free medium of 1 cm^2 to 1 ml. Inhibition of cell growth was not found after 48 h exposure. This was thought to be due to the very low concentration of toxic substances leaching from the specimens (data not shown). Therefore, an indirect contact test was adapted as described in this paper to scrutinize the toxicities of these newly synthesized hydrogels. This modified method can quantitatively illustrate the different degrees of cytotoxicity of the hydrogels. Moreover, it could be an additional assay for opaque specimens where the visual examination of cells under the sample is restricted. Cells around Cutinova Thin were found to be intact (Table I) but a suppression of cell growth was indicated by the MTT assay. This may have been due to two reasons: first, the total immersion of the sample in the medium in the indirect contact test resulted in more of the leachable products diffusing to the cells at the same time; and second, the growth medium containing the Cutinova Thin became yellowish, indicative of a pH change which may have reduced the cell growth. Ertel *et al*. [13] reported that the toxicity effect of polymers disappeared if the cells were seeded in a serum-containing medium or if concentrated solutions of proteins, such as albumin, IgG or fibronectin, were presorbed on the materials. This combined data serves to

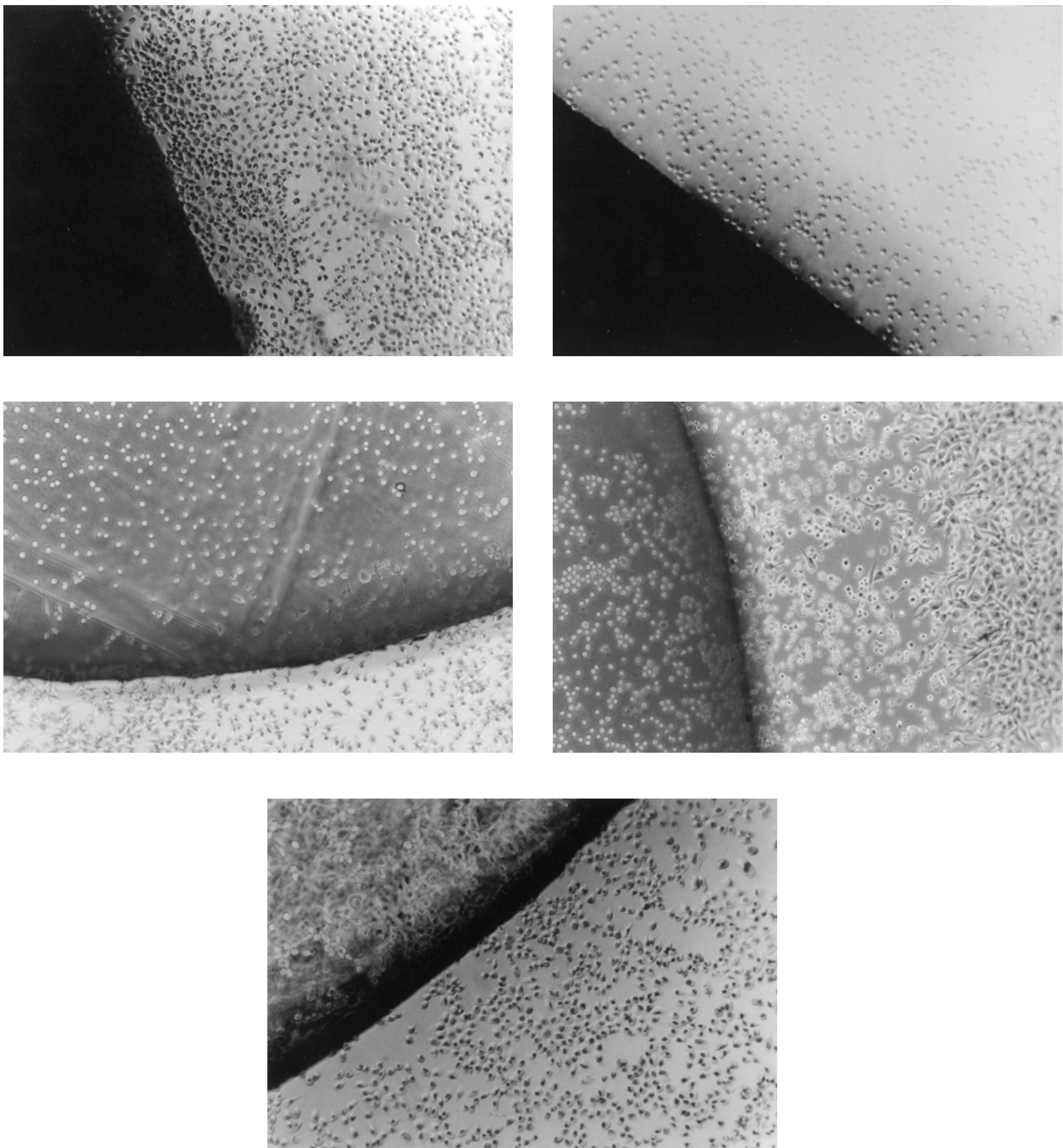

Figure 1 L929 cells in contact with (a) HDPE, negative control; (b) natural rubber, positive control; (c) PHEMA-co-10% MA, showing toxicity only under the specimen; (d) PHEMA-co-5% EA, showing extended toxicity beyond the specimen; (e) PHEMA-co-25% BA, indicating no toxic zone. Original magnification × 100.

emphasize how the methodology influences the test results. However, *in vitro* cytotoxicity tests have the advantage of being highly sensitive to any toxic substance since the many protection mechanisms in the body are absent in this case.

Structural modification of PHEMA by copolymerization with the chosen AA, MA, EA and BA, in the composition range of 5–25% by weight, altered the cytotoxicity of the hydrogel. PHEMA-co-MA containing 10–25% MA showed malformed cells under the specimens but did not exhibit statistically significant reductions in cell growth of NHF. The malformed cells found under the specimens were possibly due to the nature of the physical contact rather than leachable monomers [13]. The copolymers containing 5% of the alkyl acrylates were confirmed to be more cytotoxic to both the L929 cells and NHF than PHEMA $(p < 0.05)$. However, the toxicity decreased when the alkyl acrylate content increased, particularly in the cases of EA and BA. The glass transition temperatures (T_g) of PEA and PBA were found to be approximately -17 and $-46\,^{\circ}\mathrm{C}$ respectively, while that of PHEMA was around $109\,^{\circ}\mathrm{C}$. Consequently, both PEA and PBA are soft and flexible at room temperature whereas PHEMA is hard and brittle. In addition, PEA and PBA films have sticky surfaces when dry but lose this stickiness when wet. This would tend to render them non-adherent to the skin and therefore unsuitable for use as wound dressings. After careful consideration of all the results, P(HEMA-co-EA) with EA contents of 15–25% and P(HEMA-co-BA) with BA

T A B L E I Cytotoxicity of the various hydrogels to L929 cells from the direct contact test

Sample	Description of reactivity	Reactivity (USP 24)
PHEMA	Some malformed cells under specimen and toxic zone $\approx$ 1 mm.	Mild–moderate
PMA	Some malformed cells in some areas under specimen but no toxic zone	Slight
P(HEMA-co-5%MA)	Some malformed cells under specimen but toxic zone $\approx$ 0.4 mm.	Mild–moderate
P(HEMA-co-10%MA)	Some malformed cells under specimen but no toxic zone	Mild
P(HEMA-co-15%MA)	Some malformed cells under specimen but no toxic zone	Mild
P(HEMA-co-20%MA)	Some malformed cells under specimen but no toxic zone	Mild
P(HEMA-co-25%MA)	Some malformed cells under specimen but no toxic zone	Mild
PEA	No detectable toxic zone around or under specimen	None
P(HEMA-co-5%EA)	Toxic zone $\approx$ 3.6 mm. beyond specimen	Moderate
P(HEMA-co-10%EA)	Some malformed cells under specimen but no toxic zone	Mild
P(HEMA-co-15%EA)	No detectable toxic zone around or under specimen	None
P(HEMA-co-20%EA)	No detectable toxic zone around or under specimen	None
P(HEMA-co-25%EA)	No detectable toxic zone around or under specimen	None
PBA	No detectable toxic zone around or under specimen	None
P(HEMA-co-5%BA)	Toxic zone $\approx$ 4.6 mm. beyond specimen	Moderate
P(HEMA-co-10%BA)	No detectable toxic zone around or under specimen	None
P(HEMA-co-15%BA)	No detectable toxic zone around or under specimen	None
P(HEMA-co-20%BA)	No detectable toxic zone around or under specimen	None
P(HEMA-co-25%BA)	No detectable toxic zone around or under specimen	None
Cutinova Thin	No toxic zone, cells not visible under opaque specimen	N/A
HDPE	No detectable toxic zone around or under specimen	None
Natural rubber	Toxic zone $\approx$ 5.3 mm. beyond specimen	Moderate

N/A = Not applicable.

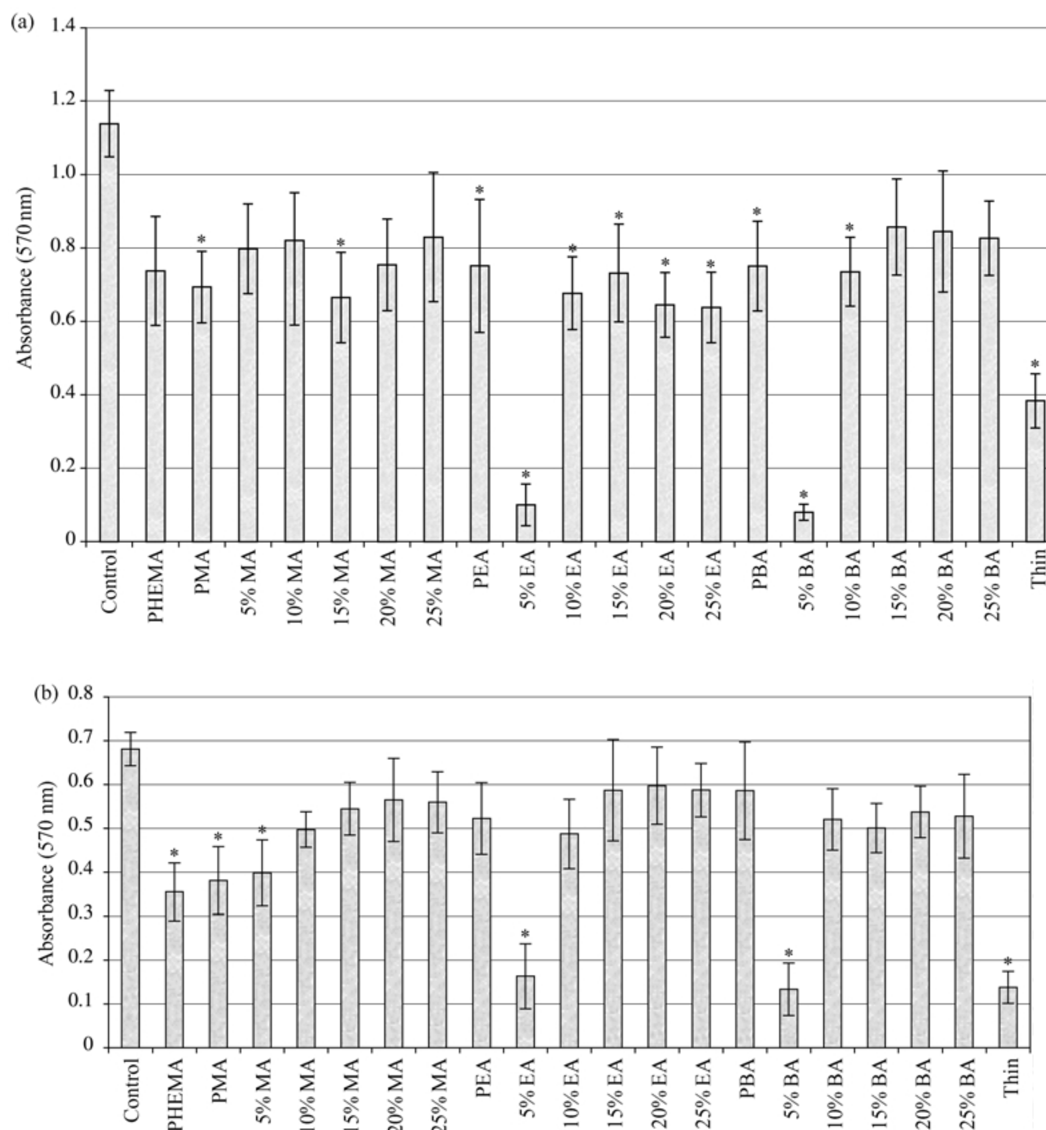

Figure 2 Cytotoxicity of hydrogels using MTT assay with (a) L929 cells, and (b) normal human fibroblasts. The results are expressed as mean values and 95% CI for the means ($n \geq 6$). * Indicates statistical difference from the controls ($p < 0.05$).

contents of 10–25% would appear, on the basis of their non-cytotoxicity, to be the most attractive for further specific biological study with a view to their potential use as temporary skin substitutes. Additional parameters which need to be monitored include cytoskeleton, cell signaling, protein synthesis and cytokine levels. Human keratinocytes and endothelial cells involved in the re-epithelialization and wound healing processes should also be employed in addition to the normal human dermal fibroblasts.

Acknowledgments

The authors wish to thank Dr. Patricia Watts of the Animal Cell Culture Laboratory at BIOTEC and Dr. Somporn Sawasdison of the Department of Pathology, Faculty of Dentistry, Chulalongkorn University, for their helpful advice and assistance.

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Received 8 August 2002
and accepted 4 February 2003