The development of *in vitro* biocompatibility tests for the evaluation of intraocular biomaterials

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Recent developments in ocular implant technology require the *in vitro* evaluation of ocular compatibility in early stage development programs. This requires an understanding and appreciation of the biological interactions which occur in the ocular environment and their relevance with respect to the clinical complications associated with surgical implantation of devices. This paper describes the development of a series of clinically reflective *in vitro* assays for assessing the potential ocular compatibility of novel intraocular lens materials. *Staphylococcus epidermidis* attachment, fibrinogen adsorption, mouse embryo fibroblast 3T3 adhesion and proliferation, primary rabbit lens cell adhesion, human peripheral blood macrophage adhesion and granulocyte activation tests were employed to evaluate two widely used intraocular biomaterials poly(methyl methacrylate) (PMMA) and silicone, and a novel biomimetic phosphorylcholine-based coating (PC). The performance of these materials in the *in vitro* assays was compared to their ability to reduce postoperative inflammation *in vivo* in a rabbit model. The results demonstrated that the *in vitro* assays described here are predictive of *in vivo* ocular compatibility. These assays offer a more relevant means of assessing the ocular compatibility of biomaterials than those presently required by the authorities for regulatory approval of medical devices and implants.

1. Introduction

Historically, the selection of biomaterials for use in ocular implants has been primarily concerned with the physicochemical and optical properties of the material. Biological assessment of material performance has often been restricted to tests, such as *in vitro* toxicity of extracts, *in vivo* sensitization tests and muscle implantation tests, required by the regulatory authorities for approval of medical devices and implants. Although these tests provide useful indicators of possible cytotoxic leachates and gross tissue incompatibility their relevance to the ocular environment is questionable.

The recent recognition that biocompatibility is important in relation to the longevity of ocular implants has led to an increasing need for clinically reflective bioassays for the assessment of ocular compatibility [1]. Such bioassays have particular application in the screening of novel materials as an integral part of development material programs for intraocular lenses, phakic intraocular lenses, glaucoma drainage devices, intrastromal lenses, keratoprostheses and contact lenses. The development of clinically reflective *in vitro* assays which will inform appropriately on the *in vivo* performance requires an understanding of the biological interactions dominant in the ocular environment and the role of these interactions in the clinical complications associated with device implantation. This paper focuses on the development of a series of clinically reflective *in vitro* assays for the assessment of ocular compatibility of novel intraocular lens materials.

The major clinical complications associated with cataract extraction and intraocular lens implantation are highlighted in Table I [2]. Postoperative inflammation occurs after surgery and can be primarily attributed to surgical trauma and disruption of the blood–ocular barrier during surgery [3]. However, the inflammatory process may lead to increased inflammatory cells in the aqueous which may adhere to the surface of the intraocular lens causing enhanced inflammatory response through the release of proinflammatory mediators. The adhesion of cells to the lens is facilitiated by the initial adsorption of blood-aqueous protein to the lens surface particularly fibrinogen and vitronectin [4]. The assessment of both protein adsorption and the adhesion and activation of macrophages and neutrophils is therefore a primary indicator of the propensity of a material surface to contribute to this inflammatory process.

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TABLE I Clinical complications following cataract surgery and intraocular lens implantation and associated *in vitro* assays

Clinical complication	Relevant *in vitro* assay
Postoperative inflammation	Protein adsorption 3T3 fibroblast adhesion Macrophage adhesion Granulocyte adhesion and activation
Postoperative endophthalmitis	Bacterial adhesion
Corneal decompensation	Corneal touch test
Posterior capsule opacification	Lens epithelial cell adhesion Protein adsorption 3T3 fibroblast adhesion Macrophage adhesion Granulocyte adhesion and activation

Although postoperative bacterial endophthalmitis occurs in less than 0.1% of clinical cases of cataract extraction it may be catastrophic [5], leading to blindness in a high percentage of cases. The causative organisms are primarily *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Propionibacterium acnes* which are transfered from the patient to the ocular environment during intraocular lens implantation. Adhesion of microorganisms to the lens surface is therefore important with respect to the potential for the material to facilitate transport of microorganisms into the eye [6, 7].

Corneal endothelial cell damage and the associated corneal decompensation has been reduced in recent years by the use of viscoelastic agents during surgery and the preferential use of posterior chamber intraocular lenses which are unlikely to interact with the cornea following implantation [8]. However accidental contact between the intraocular lens and the corneal endothelium during implantation may still result in corneal damage [9]. Traditionally, corneal endothelial cell damage has been assessed by placing the material in contact with a rabbit corneal button and assessing the gross contact damage [10]. This paper reports an alternative method using cultured bovine corneal endothelial cells which offer a more clinically reflective means of assessing the tendency of a material to cause corneal endothelial cell damage on contact adhesion.

Finally posterior capsule opacification (PCO) or secondary cataract is considered to be the major long-term complication of cataract surgery [11]. Recent work has shown that the major cause of PCO is the migration of lens epithelial cells across the posterior surface of the capsular bag [12]. Although the migration of lens cells across the surface of intraocular lenses tends to be slower than across the capsular bag this may also contribute to the secondary visual impairment [13]. Adhesion of lens epithelial cells to both the intraocular lens and capsular bag at the lens periphery may also contribute to the contact inhibition of epithelial cell proliferation across the posterior capsular bag observed following implantation of particular intraocular lenses [14]. The assessment of lens epithelial cell adhesion and proliferation on material substrates may therefore provide some indication of whether the material is likely to promote PCO. As PCO *in vivo* has been shown to be greatly stimulated by intraocular inflammation [15], the adhesion of proteins and inflammatory cells to the lens material may also be an important indicator of the rate of secondary cataract formation. However, the full relevance of these factors to the rate and extent of secondary cataract formation have yet to be evaluated.

This paper reports the development of two types of *in vitro* assays. The Phase I assays, which assess protein adsorption, fibroblast adhesion and bacterial adhesion, were designed to provide rapid *in vitro* screens of generic biocompatibility for the evaluation of a range of primary materials. The Phase II assays are more specific to the ocular environment, are more clinically reflective and are designed for the late stage evalution of new intraocular materials. The paper describes the use of these assays to evaluate the *in vitro* biocompatibility of two intraocular biomaterials poly(methyl methacrylate) (PMMA) and silicone) and a novel biomimetic phosphorylcholine-based coating (PC). The results of the *in vitro* studies have been compared to the *in vivo* performance of intraocular lenses fabricated from these materials in a rabbit model.

2. Materials and methods

PMMA sheets of uniform 1 mm thickness were prepared by solvent evaporation of a 10% (w/v) solution of PMMA in Analar grade dichloromethane. For the *in vitro* studies discs of material were cut from medical grade silicone and PMMA sheets using standard cork borers. All discs were sterilized using ethanol and stored under sterile conditions. PC-coated discs were prepared by dip-coating discs of PMMA or silicone with a 0.5 mg ml^{-1} solution of a 2-methacryloyloxyethyl phosphorylcholine containing copolymer supplied by Biocompatibles Ltd in Analar grade ethanol. The PC discs were air-dried under sterile conditions for 10–15 min at room temperature and subsequently stored under sterile conditions.

For the *in vivo* studies PMMA and silicone intraocular lenses were supplied by ChironVision UK. PMMA lenses were coated with the a phosphorylcholine-methacrylate copolymer, as described above and ethylene oxide sterilized.

2.1. Phase I *in vitro* assays

2.1.1. Adsorption of fibrinogen

The relative levels of fibrinogen adsorption to the materials were determined using a three-stage enzyme-linked immunosorbent assay (ELISA). Discs of each material (0.21 cm^2) were incubated with 100 ng of human fibrinogen in 50 µl of phosphate-buffered saline (PBS) pH 7.4 (all from Sigma Chemical Co.). The incubation was carried out for 2 h at room temperature. Following adsorption the samples were extensively washed with PBS and excess non-specific binding sites on the surfaces were blocked by incubation with a 4% (w/v) solution of bovine serum albumin (BSA) (Cohen fraction V, Sigma) overnight at 4 °C. Following further washing in PBS the samples were incubated for 30 min with a polyclonal goat antiserum directed against human fibrinogen and diluted 1 : 1000 in PBS. The samples were subsequently

washed and the procedure was repeated using sequential 1 : 500 dilutions of a secondary and tertiary antibody (rabbit antigoat and goat antirabbit) conjugated to horseradish peroxidase (HRP). Antibody binding was visualized using the turnover of the HRP-specific chromogen 2,2′ azino-bis(3-ethylbenzthioline-6-sulfonic acid) (ABTS) in a standard phosphate–citrate assay buffer containing urea and H_2O_2. Non-specific binding was assessed by using the ELISA in the absence of fibrinogen.

2.1.2. Adhesion of 3T3 mouse embryo fibroblasts

The immortal mouse embryo fibroblast cell line 3T3 was routinely cultured in Dulbecco's modification of Eagles medium (DMEM, Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco) at a density of 6000 cells cm^{-2} at 37 °C in 5% CO_2. Cells were passaged every 3 days by standard dispersion using trypsin–ethylenediamine tetra-acetic acid (EDTA) solution (0.25% Gibco). Viability was routinely checked by trypan blue counting and was always in excess of 90%. To determine the adhesion of 3T3 fibroblasts to the materials, 6000 cells cm^{-2} were seeded onto 1 cm^2 discs of each material in sterile 24-well tissue culture plates. The cells were allowed to attach and divide for 72 h under normal growth conditions. The discs and their adherent cells were then removed, washed with PBS and fixed using 3.7% (v/v) paraformaldehyde in PBS. The number of adherent cells was determined by scoring the number of cells present in 50 randomly selected fields using light microscopy and image analysis.

2.1.3. Bacterial adhesion

S. epidermidis strain 901, a clinical isolate, was grown in Nutrient Broth Oxoid No. 2 broth at 37 °C in 5% CO_2. Bacterial cells were pelleted in a refrigerated centrifuge and washed three times in sterile PBS. The cells were resuspended in PBS and the optical density at 540 nm measured and the bacterial cell concentration adjusted to 3×10^8 cells ml^{-1}. Discs (1 cm^2) of the materials were incubated with the resultant bacterial suspension for 4 h at 37 °C with constant agitation. The test samples were then removed, washed and the ATP extracted from the adherent bacteria using a commercial luminometry lysis buffer (0.1 M Tris–acetate pH 8, 2 mM EDTA containing 0.1% trichloroacetic acid). The amount of ATP in each sample was measured using a commercial kit (BioOrbit-Wallac, Turku, Finland) and a 96-well plate luminometer (Amerlite, Amersham). The numbers of adherent bacteria are directly proportional to the relative light units measured for the released cellular ATP [16].

2.2. Phase II *in vitro* assays

2.2.1. 3T3 Fibroblast proliferation

3T3 fibroblasts were cultured as described above and plated onto 1 cm^2 discs of material at 6000 cells cm^{-2}. 72 h after passaging the materials were incubated for 24 h with complete growth medium containing 25 mM bromodeoxyuridine (BrdUrd) in the presence of 2 mM fluorodeoxyuridine to inhibit thymidylate synthetase. The adherent cells were then washed three times in PBS and fixed with 3.7% (w/v) paraformaldehyde at room temperature for 10 min. The fixed cells were subsequently permeabilized by incubation with a 1% (v/v) solution of Triton X-100 for 2 min. The cells on the discs were then incubated for 90 min with 15 ml of a prediluted commercial solution containing a mouse monoclonal antibody to BrdUrd and a nuclease to denature cellular DNA (Amersham). The cells were then washed a further three times in PBS and nuclei which had incorporated cytochemically detectable quantities of BrdUrd were visualized using an avidin–biotin peroxidase ''RapidStain'' kit. The labeling index of the cells on each sample was determined by scoring 200 positive or 1000 total cells in randomly selected fields.

2.2.2. Rabbit lens cell adhesion

AGO4677, a mortal primary rabbit lens epithelial cell strain was obtained from the National Institute of Aging (NIA) repository (Cambden, NJ, USA). The cells were cultured in minimal essential Eagle's medium (MEM) with double the normal concentration of non-essential amino acids (Gibco) and 10% (v/v) FCS at a density of 6000 cells cm^{-2} at 37 °C in 5% CO_2. The cells were never permitted to become confluent under maintenance conditions and were passaged by routine trypsin dispersion. Adhesion of the AGO4677 was assayed by ATP extraction 72 h after 1000 viable rabbit lens cells were plated on to discs of material. ATP was liberated by incubating each disc with 100 µl of a sterile hypotonic lysis buffer (0.01 M Tris–acetate pH 8, 2 mM EDTA). The ATP solution was then diluted 1 : 1 with a commercial assay buffer designed for ATP luminometry (0.1 M Tris–acetate pH 8, 2 mM EDTA) and the amount of ATP in each sample measured using a commercial kit (BioOrbit-Wallac, Turku Finland) and a 96-well plate luminometer (Amerlite, Amersham).

2.2.3. Macrophage adhesion

Aliquots of human peripheral blood (20 ml) were withdrawn from a healthy male volunteer and the blood defibrinated by agitation with sterile glass beads. Following removal of the fibrin clot the blood was diluted 1 : 1 with RPMI 1640 medium (Gibco) and the mononuclear cells (MNCs) purified by sedimentation on a commercial Ficoll gradient (Lymphoprep, ICN-Flow). After sequential centrifugation at 400 ***g*** for 5 min to remove residual Lymphoprep the MNCs were resuspended in sterile macrophage serum-free medium (Gibco) and counted using a haemocytometer. 2×10^5 MNCs (2×10^5) were plated onto each of a series of 1 cm^2 discs of the materials and left to adhere overnight in a sterile 12-well tissue culture dish in a 37 °C incubator under 5% CO_2. The discs were then each washed three times with PBS and fixed in 4% (w/v) paraformaldehyde for 10 min at room temperature. The fixed cells were subsequently permeabilized by incubation with a 1% (v/v) solution of Triton X-100 for 2 min. Following quenching of endogenous residual peroxidase activity by a 72 h incubation in 9% H_2O_2 and extensive washing in

PBS a 1 : 100 dilution of a mouse antimacrophage monoclonal antibody (Dako Ltd, diluted in PBS + 1% FCS) was added to each disc and allowed to incubate overnight at 4 °C in a humidified chamber. The discs were then washed a further three times in PBS and macrophages visualized using ''RapidStain'' kit (Sigma). The number of macrophages was determined in 50 randomly selected fields.

2.2.4. Granulocyte adhesion and activation

40 ml of venous blood was obtained as described above, defibrinated, and the blood diluted 1 : 1 with RPMI 1640 medium. Granulocytes (PMNs) were purified using LymphoSep, diluted and washed three times with RPMI 1640 medium. The cells were resuspended in 5 ml RPMI 1640 medium supplemented with 10% FCS, 1% antibiotic solution and 1% glutamine. The cell suspension was counted using a haemocytometer and adjusted to 1×10^6 cells ml^{-1}. Discs (1 cm^2) of the materials were incubated for 30 min with 1×10^6 PMNs for 60–90 min in an incubator at 37 °C containing 5% CO_2. The materials were then gently washed in PBS to remove non-adherent cells and the adherent cells incubated with a commercial solution of nitrotoluene blue for 90 min at 37 °C. The discs of material were viewed under light microscopy and the mean numbers of adherent cells and activated granulocytes determined by scoring 30 randomly selected fields of view.

2.2.5. Corneal endothelial touch test

The corneal endothelial touch test utilizes the BCE/D bovine corneal endothelial cell line. BCE/D has an indefinite growth potential and expresses a similar spectrum of function-related enzymes to those found in the normal human cornea. Cultures of BCE/D corneal endothelial cells were grown to confluence in six-well tissue culture plates. The cells were medium changed to remove cell debris and allowed to recover for 24 h. The medium was then removed and 1 cm^2 discs of material were placed gently on the surface. Contact pressure was applied using a 2.4 g mass acting under gravity. Pressure was maintained for 5 min. Following the removal of the test material the monolayers were covered in fresh medium, allowed to recover for 30 min in an incubator at 5% CO_2 and 37 °C and then processed for photography. The degree of damage was subjectively assessed on a numerical scale of 0–10 with a score of 0 representing minimal damage and a score of 10 representing complete destruction of the cell monolayer.

2.3. *In vivo* studies

New Zealand White rabbits were anaesthetized using general anaesthesia and the natural crystalline lens removed from one eye of each rabbit using phacoemulsification. PMMA, PC-coated PMMA and silicone lenses $(n > 6)$ were implanted into the capsular bag through a 6-mm incision and the incision sutured using a single nylon suture. The rabbits were allowed free access to food and water following surgery and were monitored on a daily basis. The inflammatory response, in terms of anterior flare and anterior cells, and the deposition of cells on the lens surface were assessed at 1 week, 2 weeks and 4 weeks by slit-lamp examination. Giant cell number was evaluated clinically by two independent observers. Diffuse cell adherence was graded by the same observers using an arbitrary scoring system ranging from 0 (none) to 5 (extremely severe). All examinations by the observers were scored blind.

This study was conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

3. Results and discussion

The results of the use of the Phase I and II *in vitro* assays for the evaluation of the potential ocular compatibility of PMMA, silicone and PC-coated substrates are shown in Figs 1 and 2, respectively. The greater adsorption of fibrinogen (Fig. 1a), adhesion of fibroblasts (Fig. 1b), bacteria (Fig. 1c), lens epithelial cells (Fig. 2a) and macrophages (Fig. 2c); activation of granulocytes (Fig. 2d), and tendency to support the proliferation of fibroblasts (Fig. 2b) on PMMA and silicone substrates with respect to the PC-coated materials suggest that the PC-coated material would offer improved ocular compatibility. The results of the *in vivo* studies demonstrate that this is in fact the case. There were no differences observed between the postoperative anterior flare or anterior cells for the different lenses. This is not surprising as the major contributor to these factors is the surgical intervention. However, specular comparisons of giant and diffuse cell adhesion showed a reduced cellular reaction to the PC-coated surface over the period of the study compared to commercial lens types (Fig. 3a, b). No instances of postoperative bacterial endophthalmitis or corneal endothelial damage were observed in any of the animals reflecting the quality of the surgical techniques and the relatively small sample size issued in this study. Although differences in corneal endothelial cell damage between the PMMA and silicone materials and the PC-coated materials was marked *in vitro* (Figs 2e, 3a, b) the use of a hyaluronic acid-based viscoelastic during the surgery probably served to minimize this potential problem. It should be noted that giant cell formation on the implanted intraocular lenses only occurred after a period of 2 weeks. As it is generally accepted that the giant cells on the lens surface form through the fusion of the diffuse cells, probably macrophages, it is not surprising that this delay is observed. The slower formation of giant cells on the surface of the silicone materials may reflect a difference in the ability of the cells to migrate across the lens surface and may warrant further investigation. Minimizing the initial adhesion of diffuse cells to the material surface using a PC-coating clearly helps to minimize the subsequent formation of giant cell deposit on the lens surface (Fig. 4a, b). Similar effects of intracocular lens material on the adhesion of diffuse cells and the formation of giant cells has also recently been reported in patients [17].

The biological screens have been deliberately designed to present a series of challenges of increasing

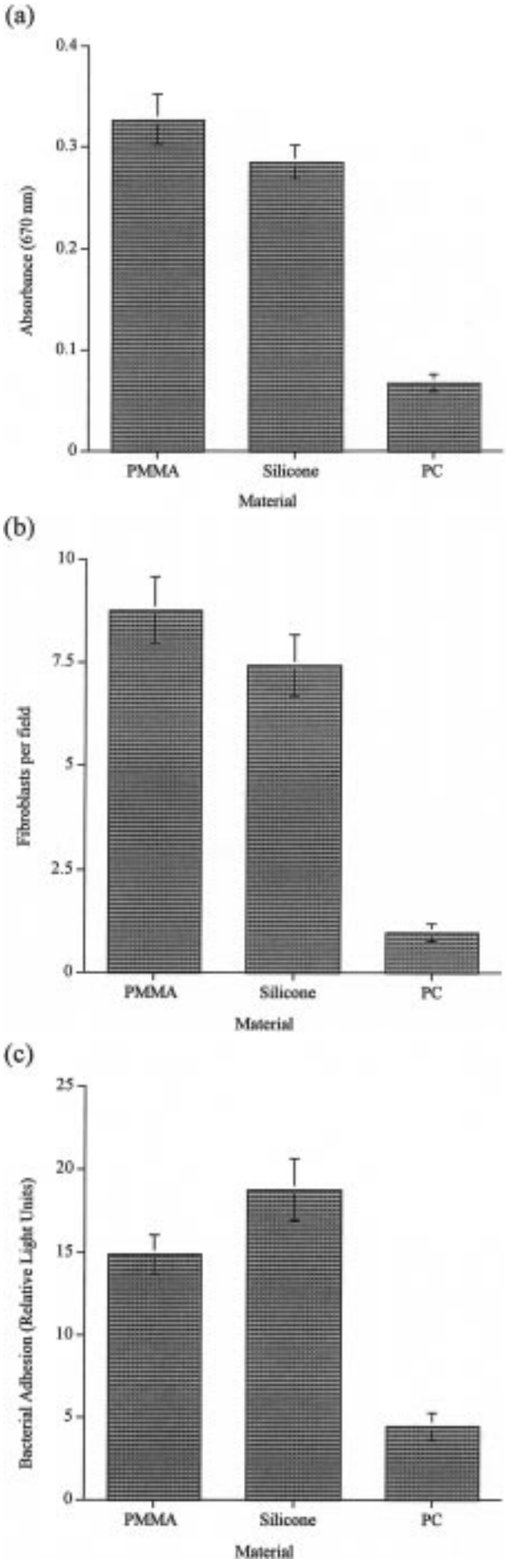

Figure 1 Phase I Assays: (a) immunoassay of fibrinogen adsorption (mean $\pm$ SEM, $n = 6$); (b) adhesion of 3T3 mouse fibroblasts (mean $\pm$ SEM, 50 fields); (c) adhesion of *S. epidermidis* 901 (mean $\pm$ SEM, $n = 6$) to PMMA, silicone and PC-coated substrates.

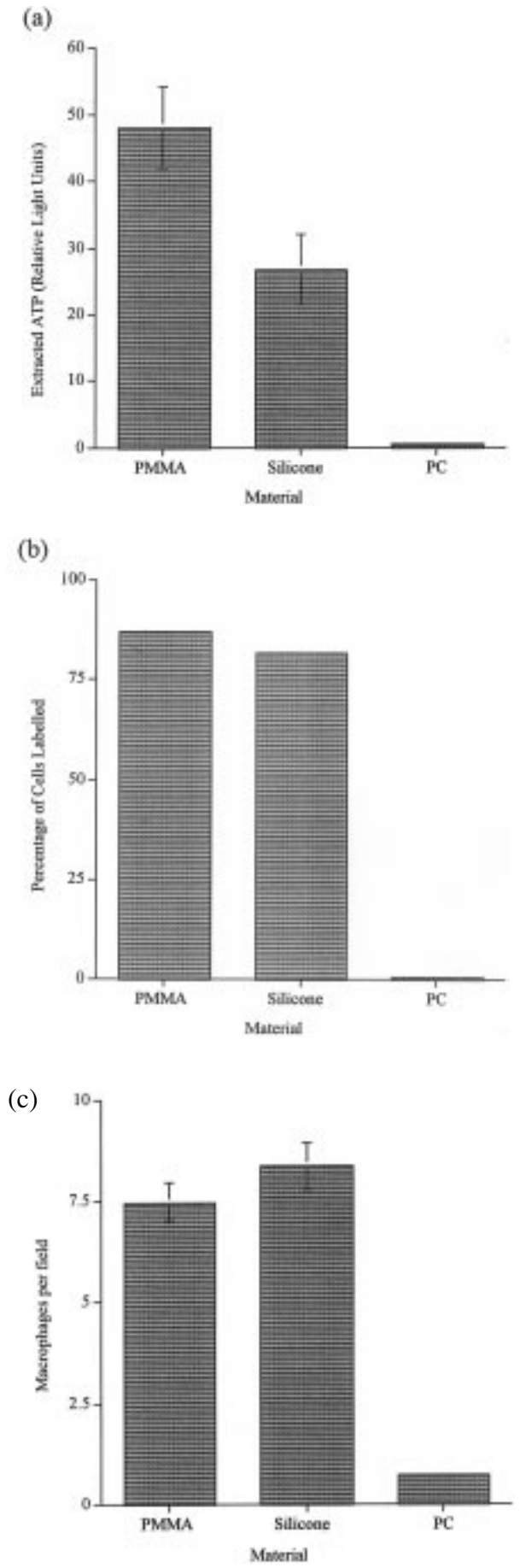

Figure 2 Phase II Assays: (a) rabbit lens epithelial cell adhesion (mean $\pm$ SEM, $n = 6$); (b) S-phase indices for proliferating fibroblasts; (c) adhesion of macrophages visualized by immunocytochemical staining (mean $\pm$ SEM, 50 fields); (d) granulocyte activation (mean $\pm$ SEM, 30 fields); (e) corneal endothelial cell damage for PMMA, silicone and PC-coated substrates.

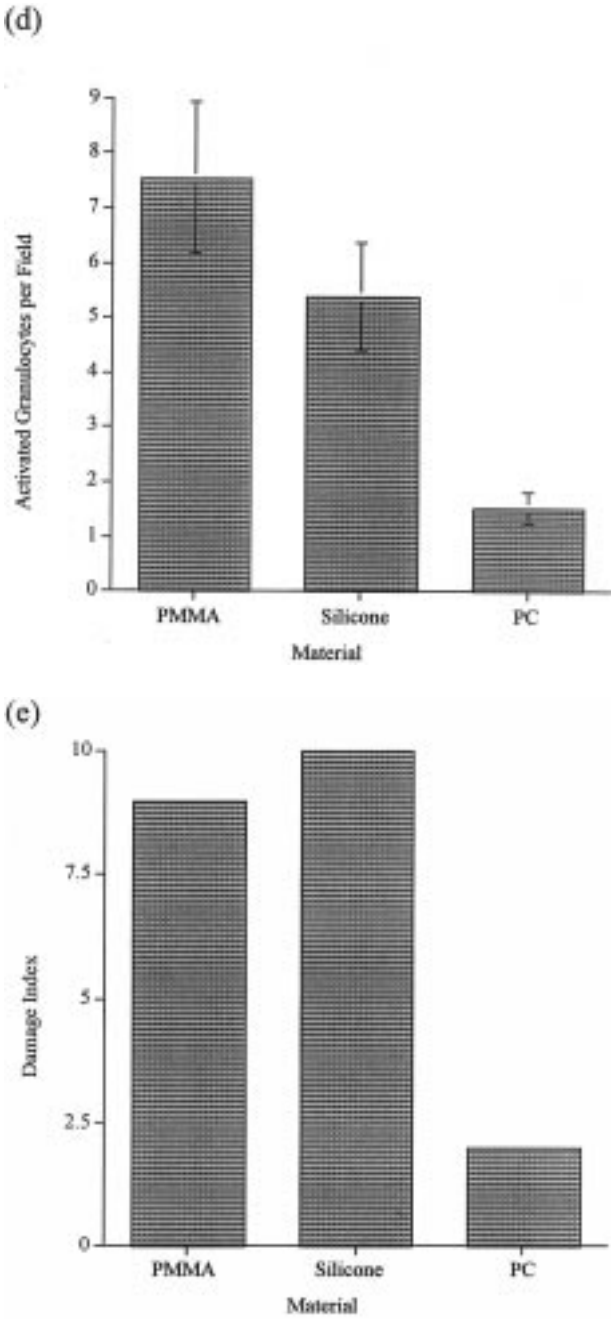

Figure 2 (Continued)

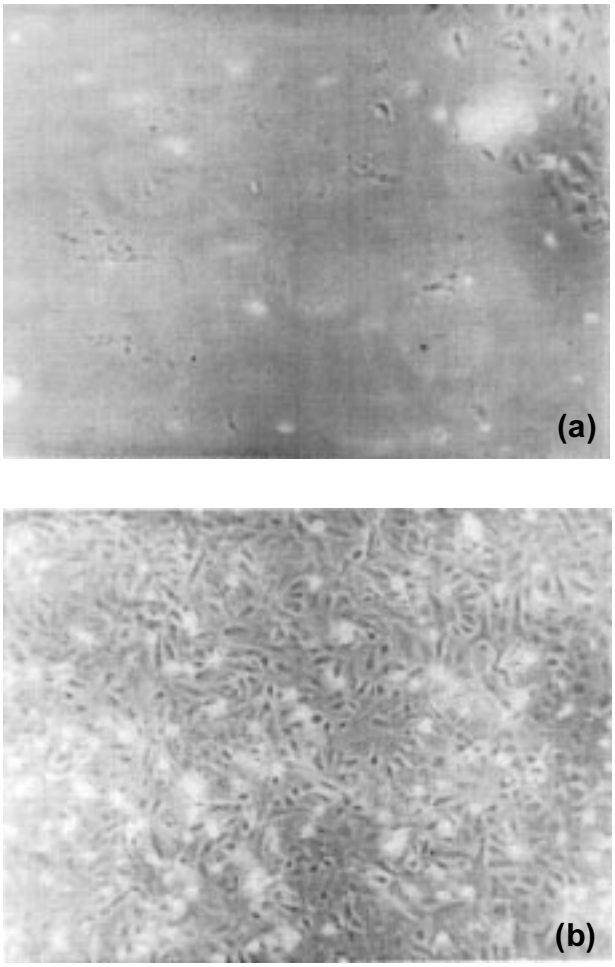

Figure 3 Photographs of bovine corneal endothelial cell damage following contact adhesion with (a) PMMA and (b) PC-coated PMMA.

sophistication and relevance to fully assess the potential biomaterials for a given application. Whilst this panel of assays has been developed to evaluate ocular biomaterials the general approach is applicable to the routine screening of materials intended for use in any *in vivo* environment. The key feature of the approach is the systematic and concerted use of multiple bioassays. This allows the screens to inform on one another and allows the elimination of test materials which fail to meet the specification with respect to biological performance at an early stage of product development.

The biological performance of the materials in the animal trials was found to be very similar to the behavior observed in the Phase I and Phase II *in vitro* studies. This strongly suggests that the *in vitro* testing regime we have developed is reflective of the *in vivo* situation. Although earlier reports have described the use of similar tests for the *in vitro* evaluation of new generation ocular biomaterials [18] and others have suggested that single *in vitro* tests could be effective substitutes for animal studies [1], we believe that our battery of clinically reflective *in vitro* tests has particular application in the early stage screening of new ocular biomaterials in new product development programs. These assays also offer a more relevant means of assessing the ocular compatibility of biomaterials than those presently required by the authorities for regulatory approval of medical devices and implants. We would therefore hope that these and other similar assays will be considered as replacements for the routine and generally inappropriate regulatory assays for the evaluation of materials for use in the ocular environment.

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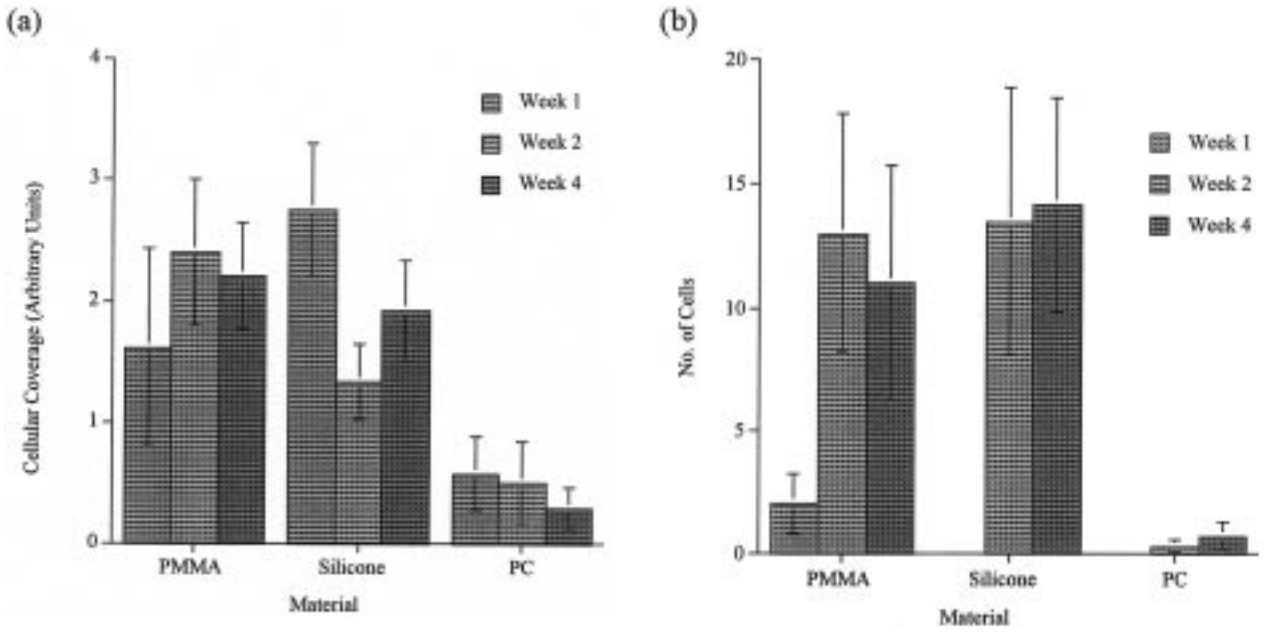

Figure 4 In vivo studies. Adhesion of (a) diffuse cells and (b) giant cells to PMMA, silicone and PC-coated PMMA IOLs after 1, 2 and 4 weeks in the rabbit model (mean $\pm$ SEM, $n = 4$-8).

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