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A MORPHOLOGICAL ASSESSMENT OF BOVINE CHONDROCYTES CULTURED ON POLY(ETHYL METHACRYLATE)/TETRAHYDROFURFURYL METHACRYLATE

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Abstract

A heterocyclic methacrylate polymer system, PEMA/THFMA, has shown potential as a biomaterial for cartilage repair in a rabbit model and has properties making it suitable for use clinically. The ability of the polymer system, PEMA/THFMA, and a related system, PEMA/HEMA, to support chondrocytes *in vitro* was assessed by scanning electron microscopy. Chondrocytes adhered to the surface of the PEMA/THFMA by day one, having a rounded morphology and clustered appearance opposed to the Thermanox® control, where the cells had spread out and become fibroblastic in appearance. The chondrocytes divided rapidly on the PEMA/THFMA system by day three and had completely covered the surface by day seven. In contrast, chondrocytes did not adhere well to the more hydrophilic PEMA/HEMA system. A few cells were seen on the surface by day one and by days three and seven, there was no evidence of cell growth or spreading across the surface.

In conclusion, the PEMA/THFMA system can support chondrocytes *in vitro*, whereas the PEMA/HEMA system does not.

Key words: Cartilage repair, chondrocytes, *in vitro*, tissue culture, biomaterial, scanning electron microscopy, poly(ethyl methacrylate), tetrahydrofurfuryl methacrylate, hydroxyethyl methacrylate.

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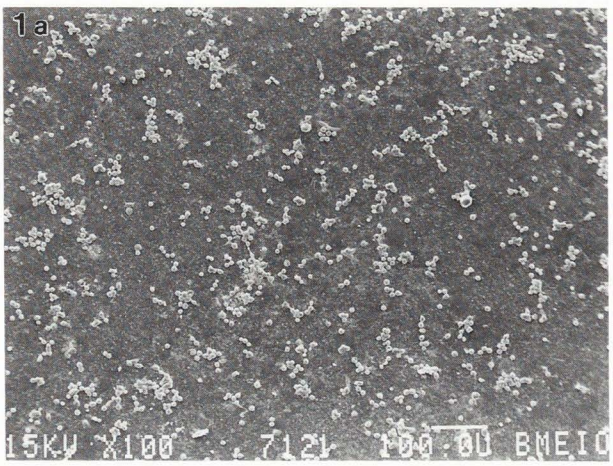
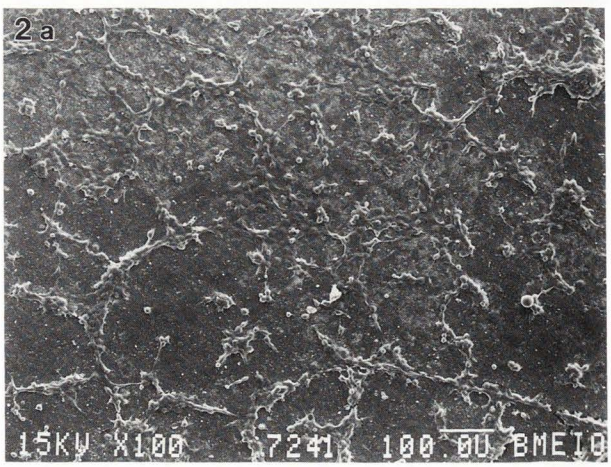
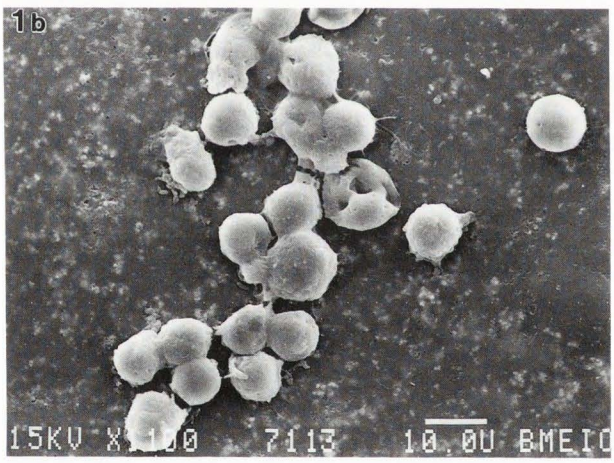
Introduction

When adult articular cartilage is damaged, repair is difficult and incomplete (Furukawa *et al.*, 1980). There have been various attempts to repair articular cartilage using biological methods such as periosteal grafts (Rubak, 1982; O'Driscoll *et al.*, 1988; Vachon *et al.*, 1991), perichondrial grafts (Homminga *et al.*, 1989; 1991; Coutts *et al.*, 1992), cartilage and chondrocyte transplantation (Aston and Bentley, 1986; Grande *et al.*, 1987; 1989) and fibrin clots (Paletta *et al.*, 1992). A variety of materials have been investigated *in vivo*, including cellophane and silicone rubber (Breck, 1967), polytetrafluoroethylene (Teflon) and polyester (Dacron) felts (Messner and Gillquist, 1993; Messner, 1994), poly(hydroxyethyl methacrylate) sponges (Kon and de Visser, 1981), collagen gels with and without chondrocytes (Wakitani *et al.*, 1989; Nixon *et al.*, 1993; Noguchi *et al.*, 1994), collagen sponges and poly(vinyl alcohol) sponges (Cobey, 1967; Speer *et al.*, 1979; Ulreich *et al.*, 1985), carbon fibres (Minns *et al.*, 1982; Robinson *et al.*, 1993), a mixture of chondrocytes and a gel-like glue containing extracellular matrix components (Itay *et al.*, 1987), a hyaluronic acid based delivery system with embedded chondrocytes (Robinson *et al.*, 1990), a poly(lactic acid) scaffold (von Schroeder *et al.*, 1991), polyurethane and a copolymer of L-lactide and caprolactone (Klompmaker *et al.*, 1992) and poly(glycolic acid) scaffolds (Vacanti *et al.*, 1991; Freed *et al.*, 1994; Rich *et al.*, 1994). The success of these methods was variable, some of the repair tissue resembling normal hyaline cartilage, but mainly, it appeared fibrous or fibrocartilaginous in nature.

In this work, a heterocyclic methacrylate polymer system, consisting of poly(ethyl methacrylate) (PEMA) polymer and tetrahydrofurfuryl methacrylate (THFMA) monomer, was investigated as a potential material for cartilage repair. It was compared to a hydrophilic variation with the monomer 2-hydroxyethyl methacrylate (HEMA) replacing the THFMA component, which has been shown to increase the rate of water uptake five-fold (Downes *et al.*, 1994a). The ability of the polymer







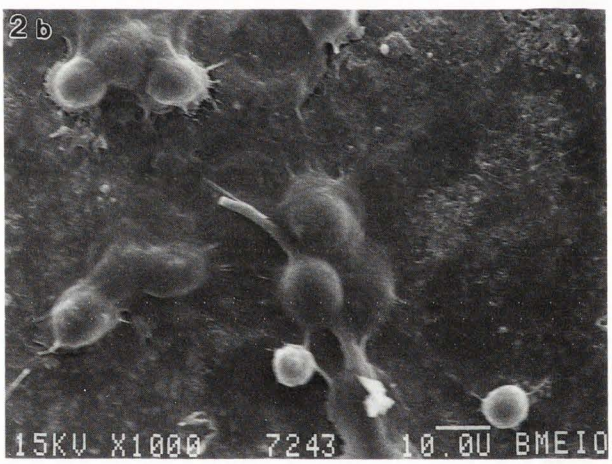


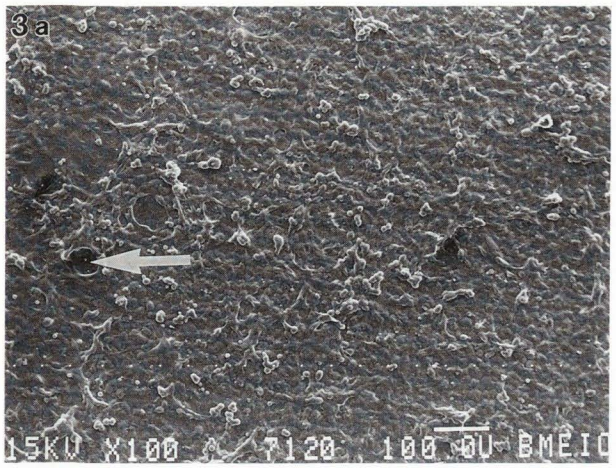
Figure 1. (**a**) Chondrocytes on the PEMA/THFMA polymer system at day one. (**b**) Higher magnification of a group of cells.

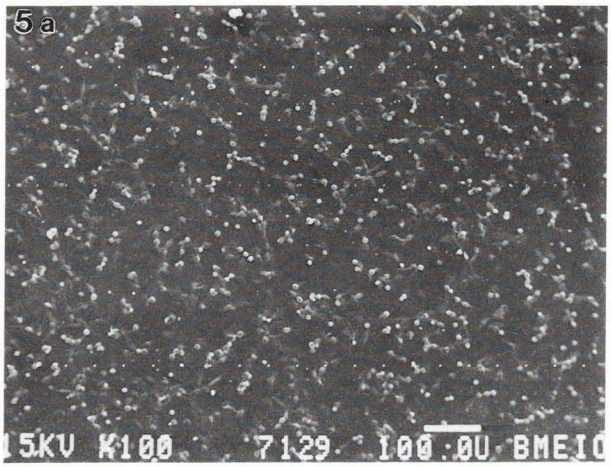
Figure 2. (**a**) Chondrocytes on the PEMA/THFMA polymer system by day three have started to grow across the surface (polymer surface indicated by P). (**b**) Higher magnification showing chondrocytes that have proliferated and started to spread.

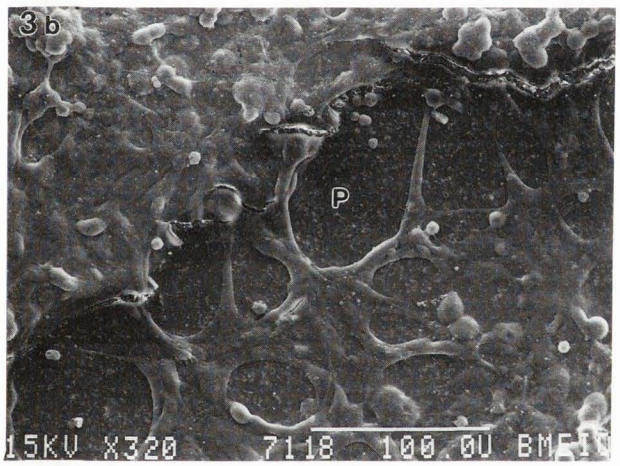
systems to support chondrocyte growth *in vitro* was assessed by scanning electron microscopy (SEM).

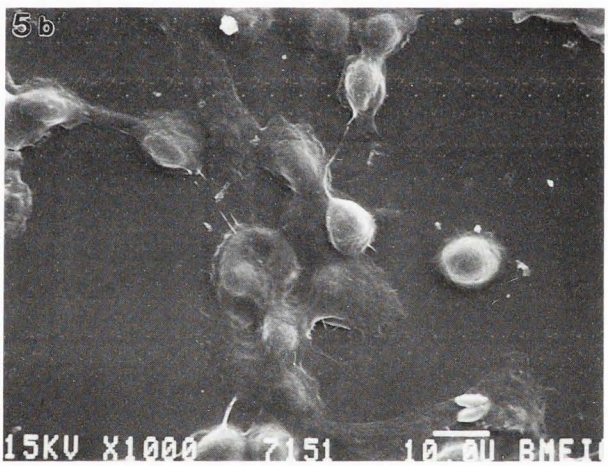
The PEMA/THFMA system is a semi-interpenetrating network with low polymerization shrinkage (Patel *et al.*, 1987). The polymerized material is cross-linked (Patel and Braden, 1989), exhibits plastic yielding and is ductile (Patel and Braden, 1991a). It is a room temperature polymerizing system with a low exothermic reaction and, therefore, is ideal for clinical applications (Patel and Braden, 1991b). The high water uptake of the system, up to 34% over two years *in vitro* (Patel and Braden, 1991c), could have advantages for cartilage repair by absorbing tissue fluid or synovium in the joint. It has also been shown to be biocompatible in the dental pulp of monkeys (Pearson *et al.*, 1987).

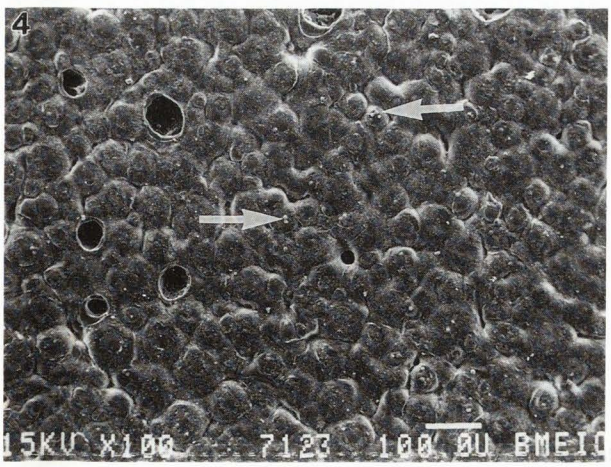
The PEMA/THFMA system has shown potential for cartilage repair in a rabbit model; as early as three weeks after implantation, a layer of fibrous and cartilaginous tissue had formed over the polymer surface. At six weeks, the resurfaced tissue was fully integrated with the adjacent normal cartilage and by eight months, remodelling of the subchondral bone was seen and the new repair cartilage remained intact (Downes *et al.*, 1994b; Reissis *et al.*, 1994a, 1994b).











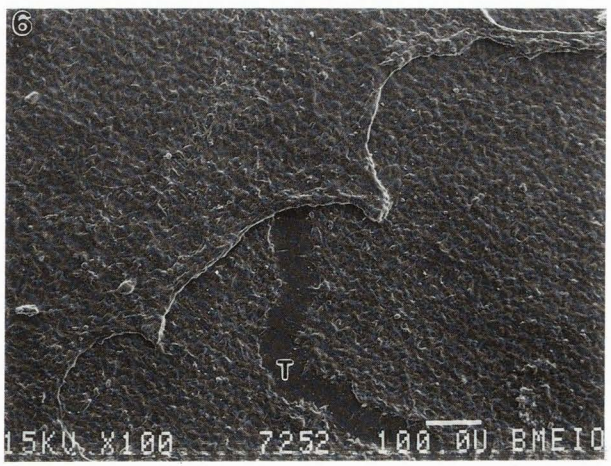


Figure 3. (**a**) Chondrocytes on the PEMA/THFMA polymer system at day seven have completely covered the surface apart from small areas (arrow). (**b**) An area where the surface has not been completely covered (polymer surface indicated by P). Note the cells have started to spread out here and become fibroblastic in appearance.

Figure 4. Chondrocytes (marked by arrows) on the PEMA/HEMA system at day one.

Figure 5. (**a** and **b**) Chondrocytes on Thermanox® at day one have adhered and started to spread out.

Figure 6. Chondrocytes on Thermanox® (T) at day seven have completely covered the surface apart from a small area.

Materials and Methods

Polymer system preparation

The PEMA/THFMA and PEMA/HEMA polymer systems were made by mixing, for one minute with a spatula, 5 g of PEMA powder (Bonar Polymers Ltd, Newton Aycliffe, U.K.) and either 3 ml of THFMA (Rohm Chemie, Darmstadt, Germany) or HEMA (Aldrich Chemical Co, Gillingham, U.K.) liquid monomer containing 2.5% v/v N,N-dimethyl-p-toluidine. The polymer mixtures were placed in a polyethylene mould at room temperature to cure, producing circular discs of approximately 13 mm in diameter and 2 mm thickness. The curing time was approximately 10 minutes and all discs were sterilized by autoclaving at 100°C for 20 minutes prior to cell culture studies.

Chondrocyte culture

Chondrocytes were obtained from bovine cartilage by a method adapted from Archer *et al.* (1990). Cartilage pieces were removed from the proximal side of an opened bovine metacarpalphalangeal joint, finely chopped and incubated with pronase type E (700 units ml^{-1}) (BDH Ltd, Poole, U.K.) in complete medium [Dulbeccos Modified Eagles medium (Gibco BRL, Paisley, U.K.) containing 20% foetal calf serum, 2% HEPES, 1% Glutamine, 10,000 units ml^{-1} Penicillin/Streptomycin and 0.85 mM ascorbic acid] for one hour followed by incubation with collagenase type 1a (300 units ml^{-1}) (Sigma, Poole, U.K.) in complete medium for two hours. The cell suspension was filtered using 70 μm cell strainers (Falcon, Becton Dickinson, Oxford, U.K.) and centrifuged at 1500 rpm for five minutes to pellet the cells. The cells were washed twice in 10 ml of complete medium and then counted using a haemocytometer. The cell concentration was adjusted to 5×10^5 ml^{-1} in complete medium and seeded directly onto either the PEMA/THFMA or PEMA/HEMA polymer system discs or Thermanox® discs (NUNC products, Life Technologies, Paisley, U.K.), as a control, in a 24-well plate. Thermanox is poly(ethylene terephthalate) extruded film approximately 0.18 mm thick that has been treated for cell attachment and radiation sterilized. The cultures were maintained in an incubator at 37°C with an atmosphere of 5% CO_2.

Scanning microscopy preparation

The polymer systems and Thermanox discs were removed at days one, three and seven and fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 hours. The discs were washed in 0.1 M sodium cacodylate buffer and stained with 0.5% aqueous toluidine blue to visualize the cells by light microscopy. The discs were then air-dried in a desiccator overnight, sputter-coated with gold and examined using a JSM-35C JEOL scanning electron microscope. Conventional SEM preparation using dehydration in alcohol could not be used, as it affected the surface of both polymer systems causing them to become softened. Freeze-drying could not be used either, as water crystals fractured the surface.

Results

The experiment was repeated 3 times and the whole area of each disc was examined. The figures shown are representative of all the samples. Chondrocytes were seen on the PEMA/THFMA disc by day 1 (Figs. 1a and 1b). These cells had a rounded morphology and appeared both singly and in clusters on the surface. Cellular processes could be seen from the chondrocytes attaching to the polymer system surface. By day three (Figs. 2a and 2b), the cells had proliferated and covered a large area of the surface. These cells were in clusters and many had a more flattened morphology compared to those at the day 1 time point. The cellular processes from the chondrocytes to the surface were also more clearly visible. The day 7 discs (Fig. 3a) showed that the chondrocytes had proliferated and covered practically the whole surface, with only small areas of the polymer system visible. Figure 3b shows one of the areas not completely covered, where the cells are very flattened and fibroblastic in appearance. Very few cells adhered to the PEMA/HEMA system at day one (Fig. 4), and even by days three and seven, there was no evidence of cell growth or cell spreading across the surface (pictures not shown). Chondrocytes adhered to the Thermanox control by day one (Figs. 5a and 5b) with many having spread out and become fibroblastic in appearance. Chondrocytes proliferated rapidly on the Thermanox and the day three (micrograph not shown) and day seven (Fig. 6) discs showed that the cells had completely covered the surface.

Discussion

Previous studies have shown that various physical and chemical properties of materials, including surface groups and hydrophilic/hydrophobic characteristics, influence cell adhesion and thus growth on the surface (Rosen and Schway, 1980; Lydon *et al.*, 1985; Dekker *et al.*, 1991; Smetana, 1993). Bagnall (1977) suggested that there may be a critical level of hydrophobicity for an implant material to be successful. As cartilage has such a high water content, between 65-85% of its mass (Jaffe *et al.*, 1974; Thompson and Robinson, 1981), a material that is more hydrophilic may be suitable for its repair. The hydrophilicity of the polymer system was,

therefore, varied in a controlled manner using the more hydrophilic monomer 2-hydroxyethyl methacrylate (HEMA) (Downes *et al.*, 1994a) to replace the THFMA component.

The PEMA/THFMA polymer system did support chondrocyte growth *in vitro* with the cells having a rounded and clustered appearance at day one, in contrast to spread chondrocytes on the Thermanox control. Cell shape is important to maintain chondrocyte phenotype (Watt and Dudhia, 1988); the fact that this polymer allows the cells, at least initially, to remain rounded, may be important in the PEMA/THFMA polymer's success as a repair material *in vivo* (Downes *et al.*, 1994; Reissis *et al.*, 1994a, 1994b). Although less so than the Thermanox control, the chondrocytes proliferated rapidly on the PEMA/THFMA system, spreading across the surface by day three and completely covering the polymer system by day seven. In contrast, chondrocytes did not adhere well to the more hydrophilic PEMA/HEMA system. A few cells could be seen on the surface at day one, however, by day three, only a couple were visible and no evidence of cell division or spreading across the surface was seen. It is possible that cells may have been lost from the surface of the PEMA/HEMA during the washing stages; if this was so, then they were only loosely attached, as all samples were prepared in the same manner.

This dramatic difference in the ability of the two polymer systems to support chondrocyte growth could be due to a variety of reasons. It could be the chemical nature of the materials, with the system containing the HEMA component unable to provide suitable support for chondrocyte adherence and growth. Cells such as fibroblasts do adhere to PEMA as a homopolymer, although they do not adhere to poly(HEMA) (Rosen and Schway, 1980; Lydon *et al.*, 1985) unless it is modified, for example, by collagen incorporation (Chirila *et al.*, 1993). In fact, poly(HEMA) is used in contact lenses because proteins and cellular material will not bind to the surfaces. It has been shown that copolymers of HEMA and ethyl methacrylate (EMA) with only 8% EMA can support fibroblasts *in vitro* (Lydon *et al.*, 1985), yet we have shown that PEMA/HEMA in the ratio of 5 g PEMA to 3 ml HEMA did not support chondrocytes. The difference in the relative hydrophilicity values of both systems could also be a factor, with the HEMA component increasing the rate of water uptake five-fold (Downes *et al.*, 1994a). Surface roughness is an important aspect for cell adhesion and it has been shown that polymers containing the HEMA component have a much smoother surface than those containing THFMA (Downes *et al.*, 1994a). There could be differences between the two systems in their abilities to bind proteins to allow chondrocyte adhesion. The PEMA/THFMA may bind proteins that maintain chondrocytes in a rounded, differentiated state, in contrast to Thermanox, where they had began to spread as early as day one. The difference in the two systems ability to support chondrocytes is unlikely to be due to one factor alone, but to a combination of the physical and chemical properties of the materials.

Previous work has shown that chondrocytes can be maintained in a rounded morphology when cultured in association with materials. LiVecchi *et al.* (1994) showed that chondrocytes adhered to hydrophilic and hydrophobic high density polyethylene (HDPE) porous substrates. The HDPE substrates allowed the maintenance of differentiated chondrocytes compared to cells allowed to adhere to flat culture plates. The hydrophilic substrates contained cells with significantly more type II collagen than the hydrophobic substrates, showing the hydrophilicity to contribute positively to the maintenance of the chondrocytic phenotype. However, after 21 days in culture, the cells had spread on all the substrates. Freed *et al.* (1993) investigated neocartilage formation *in vitro* using chondrocytes cultured on biodegradable poly(lactic acid) and poly(glycolic acid) scaffolds. The chondrocytes did not divide within the first three days of culture, in contrast to our system where rapid division occurred within this period. The cells remained rounded on the poly(glycolic acid) and reached a cell density at six weeks that was eight fold higher than at day one. The cells were spindle-shaped on the poly(lactic acid) and it was less successful at supporting chondrocyte growth. The biodegradation rates influenced the success of the scaffolds, a consideration not needed for the non-degradable PEMA/THFMA described here. Nixon *et al.* (1993) used porous collagen matrices to support chondrocytes *in vitro*. The chondrocytes in the interstices of the implant were of a rounded morphology and proliferated slowly, however the chondrocytes on the surface proliferated faster, dedifferentiated and became spindle shaped in appearance. Macroporous semi-interpenetrating network hydrogels, made to mimic the mechanical and structural properties of cartilage, have shown potential for support of chondrocytes *in vitro*. By varying the pore size of the hydrogel, it is possible to maintain rounded chondrocytes. If the pores are too small or too large the chondrocytes spread out, dedifferentiate and form a monolayer. It was found that a pore size of 38 μm was optimal, discouraging spreading and allowing the cells to remain rounded for several days, although there was a low rate of replication (Corkhill *et al.*, 1993).

The polymer system we describe here, PEMA/THFMA, has advantages for clinical use as it is easy to handle and as it polymerizes *in situ* there is no problem with anchorage and little chance of it becoming loose

and unstable. In conclusion, this study has shown that the PEMA/THFMA system can support chondrocytes *in vitro*, whereas the PEMA/HEMA system does not.

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Discussion with Reviewers

H.W.B. Jansen: Terms as "semi-interpenetrating network" leave too much to my imagination.
Authors: The term "semi-interpenetrating network" has been used by other authors (Corkhill *et al.*, 1993). It is used here to describe the PEMA/THFMA system as the PEMA dissolves in the THFMA monomer component and the THFMA polymerizes around it. It is not a totally homogeneous material, as the PEMA does not completely dissolve.

K. Draenert: A statistical evaluation method must be included.
Authors: This was a morphological assessment and no quantitative work was done, so we cannot do any statistical analysis.

K. Draenert: Scanning electron microscopy of the non-autoclaved material and autoclaved one should be investigated. All methacrylates are thermoplastics and will drastically change their shape after such procedure.
B.J. Tighe: Does the clarity and nature of the materials change when autoclaved? (This might provide evidence relating to physical homogeneity).
Authors: We obtained micrographs of this, but there was no visible difference between autoclaved and non-autoclaved samples. This is probably because the polymer is cross-linked, and although it may soften on autoclaving, it does not change shape.

K. Draenert: In **Results**, it is not mentioned why toluidine blue staining was performed.
Authors: The reason for using toluidine blue was mentioned in **Materials and Methods**. It was performed to allow visualization of the cells by LM prior to continuing to the SEM stage.

K. Draenert: Air-drying is nowadays a non-acceptable procedure for processing SEM specimens. Freeze-drying would be the method of choice to handle these methacrylate specimens.
Authors: Air-drying was shown to be the optimum method for the preparation of the PEMA/THFMA and PEMA/HEMA discs. During standard processing, the polymer system discs became softened in alcohol which distorted their surface. Freeze-drying allowed water crystal formation which fractured the surface of the polymer systems. We tried a method using hexamethyldisilazane (Nation, 1983), but this also badly distorted the surface. Attempts to use nitrous oxide preparation only resulted in oxidized material.

K. Draenert: The PEMA/HEMA scanning electron micrographs should be presented in a comparable fashion.
Authors: The PEMA/HEMA photographs are not presented in comparable fashion because, as stated in the text, there were no cells seen on the material at days 3 and 7.

K. Draenert: What exactly does "surface groups" mean?
Authors: "Surface groups" are the chemical groups present on the surface of the material.

M. LaBerge: If these materials are subjected to a high water uptake, what is the effect of autoclaving on the material properties?
Authors: Autoclaving will increase the degree of polymerization and reduce the amount of residual monomer. It might also increase the transition temperature of the polymer systems and their rigidity. They do not change shape on autoclaving probably because they are cross-linked.

M. LaBerge: Cell shape is important to maintain chondrocyte phenotype. However, it does not mean that because cells keep their "spherical shape" that they are phenotypically undifferentiated. In order to confirm this hypothesis the authors should have performed a biochemical assay to detect the presence of collagen type II or keratan sulfate or chondroitin sulfate as expected from undifferentiated chondrocytes.
Authors: The literature shows that the shape of chondrocytes determines their phenotype (Watt and Dudhia, 1988). Future work will include immunolocalization studies.

M. LaBerge: Did the authors conduct a contact angle study? This test should be performed and results included. Surface roughness has been shown by several authors to influence surface spreading and "adherence". A surface analysis of each substratum should be conducted in order to rule out the effect of surface topography on cell behavior. Parameters such as RA or CLA (roughness average), RMS (root mean square), and peak-to-valley must be evaluated prior to discussing the results. A surface analysis must be conducted in order to attribute cell behavior to chemistry of the surface. If the surface profile was not the same for all specimens (statistical significance), this discussion must take this effect into consideration.
Authors: We have not included a contact angle study. This is a new material and the research is ongoing, and this is planned for the future. A reference to the surface roughness of the materials is included in **Discussion**.

M. LaBerge: The authors could discuss the use of the PEMA/THFMA system as a bearing material for the reconstruction of focal defects. Even though chondrocytes have been shown to retain their shape on the surface of this material, what would be the effect of loading or weight bearing on the surrounding autogenous tissue?
Authors: PEMA/THFMA system is a good bearing material which has been shown to be successful in a rabbit model after a period of two years.

D.A. Grande: The PEMA/THFMA is a semi-interpenetrating network. How large are the pores?
Authors: Although the PEMA/THFMA is a semi-interpenetrating network (SIPN), it does not really have pores. When water is taken up into the system, channels are then formed.

D.A. Grande: It is quite well accepted that cartilage is a communicating network with regards to solute diffusion. How would this system be integrated in an *in vivo* site and would it inhibit diffusion to chondrocytes?
Authors: It integrates very well *in vivo* as it polymerizes *in situ* and swells slightly. Follow up studies of 8 months in a rabbit model show good cartilage repair in defects with the PEMA/THFMA system (Downes *et al.*, 1994b). It would not be expected to inhibit diffusion to chondrocytes as the material has a high water uptake (Patel and Braden, 1991c), and bovine serum albumin and growth hormone incorporated into the system have been shown to be released during *in vitro* studies (Di Silvio *et al.*, 1994).

D.A. Grande: How close are the mechanical properties of this polymer to native cartilage with respect to modulus and apparent permeability?
Authors: The modulus value of the PEMA/THFMA system is 1.34 GPa.

D.A. Grande: Why were these experiments not carried out longer? The time studied is rather short and longer time intervals would be of value.
Authors: It was only a seven-day experiment as we were investigating initial cell attachment and proliferation. The PEMA/THFMA discs were almost completely covered by day 7 and SEM would have revealed little more information after this time point. However, longer experiments are planned for future studies.

B.J. Tighe: Are the materials prepared for this study believed to be homogeneous or heterogeneous?
Authors: The PEMA/THFMA system is considered to be largely homogeneous as PEMA dissolves almost completely in the THFMA monomer and THFMA polymerizes around it creating a partly semi-interpenetrating network. The PEMA/HEMA is a heterogeneous material because the PEMA dissolves very little in the HEMA monomer and is in fact a mixture of PEMA surrounded by polyHEMA.

Reviewer VIII: I would recommend figures with comparable magnification, in general. In particular, I would suggest the inclusion of figures at 1000x of chondrocytes on PEMA/THFMA at day 7, on PEMA/THFMA at day 1, and on Thermanox® at day 7.
Authors: We have included a 1000x micrograph of Thermanox at day 1. We have not included other micrographs as we do not feel they will add anything to the paper since PEMA/HEMA discs showed no cells at days 3 and 7, as stated in **Results**.

Reviewer VIII: Since you address the dependence of cells attaching to a surface to the surface hydrophilicity, it would be necessary to evaluate both the numbers of adherent cells and the extent of surface hydrophilicity, e.g., the free surface energies.
Authors: We do not have data on free surface energies for the materials used in this work. This is an ongoing study and is planned for the future. As there was such a dramatic difference in the number of cells on the two polymer systems studied, we did not count the cells.

Reviewer VIII: Since the influence of surface roughness/porosity on cell attachment/cell proliferation has been discussed and since surface roughness/porosity showed significant differences in the samples used in the experiments described above, it would be necessary to evaluate the roughness/porosities and discuss their influences.
Authors: As mentioned in **Discussion**, previous work has shown that materials containing HEMA are smoother than those containing THFMA (Downes *et al.*, 1994a), although we have not conducted further investigation.

Additional Reference

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