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An *in vitro* investigation of the PEMA/THFMA system using chondrocyte culture

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Adult articular cartilage has a limited capacity for self-repair and a biomaterial to aid the process would be ideal. The polymer system, poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEMA/THFMA) has shown potential in a rabbit model for such a role. The ability of the polymer system to support chondrocytes *in vitro* was investigated by light microscopy, transmission electron microscopy and biochemical assays. The PEMA/THFMA system maintained chondrocytes in agarose in a viable state with more glycosaminoglycan (GAG) produced per unit DNA after 14 days in culture compared to the tissue culture plastic control. Chondrocytes remained rounded on the polymer system surface as opposed to well spread on the Thermanox. The PEMA/THFMA system has been shown to be biocompatible for bovine chondrocytes maintaining them in a differentiated state with enhanced GAG production.

1. Introduction

Cartilage is present at various sites in the body and consists of cells, chondrocytes, in a matrix of collagens, proteoglycans and water. There are different types of cartilage for the different support roles in the body and here we are concerned with hyaline cartilage present in joints called articular cartilage. When adult articular cartilage is damaged, by lesions or matrix break down due to disease, repair is difficult and incomplete. Total joint replacement may then become necessary using an artificial implant. A conservative surgical procedure using biomaterials to repair or support the cartilage defect would be a significant break through in orthopaedic surgery. There have been various attempts to repair articular cartilage with materials including cellophane, silicone rubber [1], polytetrafluoroethylene (Teflon), polyester (Dacron) felts [2, 3], poly(hydroxyethyl methacrylate) sponges [4], collagen gels with and without chondrocytes [5, 6], collagen sponges and poly(vinyl alcohol) sponges [7–9], carbon fibres [10, 11], a mixture of chondrocytes and a gel-like glue containing extracellular matrix components [12], a hyaluronic acid based delivery system with embedded chondrocytes [13], a poly(lactic acid) scaffold [14], polyurethane, a copolymer of L-lactide and caprolactone [15] and poly(glycolic acid) scaffolds [16–18]. The success of these materials varied, some workers reporting a repair tissue resembling normal hyaline cartilage but generally the repair appeared fibrous or fibrocartilaginous in nature. Most of the studies have been performed in animal models although there have been *in vitro* studies with chondrocytes and materials including poly(lactic acid), poly(glycolic acid) [19], collagen matrices [20, 21], macroporous semi-interpenetrating network hydrogels [22], glycolide trimethylene carbonate copolymer [23] and high density polyethylene [24]. A material for cartilage repair must be biocompatible and it is essential to consider chondrocyte shape, division and matrix production in relation to that material. In this work we report an investigation of the polymer system, poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEMA/THFMA) using two methods of chondrocyte culture; chondrocytes supported in a matrix of agarose above the polymer system and chondrocytes seeded directly onto the polymer system surface. Light microscopy was used to examine chondrocyte morphology and transmission electron microscopy (TEM) was used to assess the ultrastructure of the cells. Biochemical assessment of total deoxyribonucleic acid (DNA) and sulphated glucosaminoglycans (GAG) were measured quantitatively at seven and 14 days in culture. GAG in the extracellular matrix was visualized by staining with Safranin O.

2. Materials and methods

2.1. Polymer system preparation

The PEMA/THFMA polymer system was made by mixing, for one minute with a spatula, 5 g of PEMA powder (Bonar Polymers Ltd, Newton Aycliffe, UK) and 3 ml of THFMA (Rohm Chemie, Darmstadt, Germany) liquid monomer containing 2.5% v/v N,N-dimethyl-p-toluidine. The polymer mixture was placed in a polyethylene mould to cure at room temperature producing circular discs of approximately

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13 mm in diameter and 2 mm thickness. The curing time was approximately 10 min and all discs were sterilized by autoclaving at 100 °C for 20 min prior to cell culture studies.

2.2. Chondrocyte culture

Chondrocytes were obtained from bovine cartilage by a method adapted from Archer *et al.* [25]. 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, UK) in complete medium (Dulbeccos Modified Eagles medium) (Gibco BRL, Paisley, UK) containing 20% foetal calf serum, 2% HEPES, 1% Glutamine, 10000 units ml^{-1} Penicillin/Streptomycin, 0.85 mM ascorbic acid, for one hour followed by collagenase type 1a (300 units ml^{-1}) (Sigma, Poole, UK) in complete medium for two hours. The cell suspension was filtered using 70 μm cell strainers (Falcon, Becton Dickinson, Oxford, UK) 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. For the agarose cultures the cell concentration was adjusted to 10×10^6 ml^{-1} in complete medium and mixed with an equal volume of 4% liquid type VII agarose to give a final concentration of 5×10^6 ml^{-1} in 2% agarose and 0.5 ml plated out onto the PEMA/THFMA discs or tissue culture plastic (TCP) as a control. Chondrocytes seeded directly onto the PEMA/THFMA discs were added at a concentration of 5×10^5 ml^{-1} and Thermanox discs (NUNC products, Life Technologies, Paisley, UK) used as a control. Thermanox is polyethylene tetraphthalete 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.

2.3. GAG and DNA measurements

The agarose cultures were removed at seven and 14 days, finely chopped and digested in 1 ml buffer (9 mM cysteine hydrochloride and 2 mM diaminoethanetetraacetic acid disodium salt in PBS pH 6.0) containing 10 units of agarase (Sigma, Poole, UK) and 2 units of papain (Sigma, Poole, UK) for two days. Total DNA was measured using a fluorimetric method [26] and GAG determined quantitatively using a 1,9-dimethylmethylene blue method [27]. Pieces of agarose containing the cells were processed for histology. They were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), dehydrated through a series of alcohols to xylene then 4 changes in wax prior to embedding. Sections were cut, 5 μm thick, and stained with Safranin O to show the GAG production [28] and Mayer's Haematoxylin to stain the nuclei.

2.4. TEM preparation

Chondrocytes in agarose were processed by fixing in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), secondary fixed using 1% osmium tetroxide, dehydrated through a series of alcohols and embedded in firm Spurr's resin [29]. Ultra thin sections were cut, stained with 2% uranyl acetate and Reynold's lead citrate and examined using a Philips CM12 transmission microscope at 80 kV.

2.5. Cell attachment

Chondrocytes seeded directly onto the polymer system and Thermanox were fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), stained with toluidine blue and viewed by light microscopy.

3. Results

Viewing by TEM showed the chondrocytes to be viable in the agarose above both the TCP and the PEMA/THFMA system. Fig. 1 and Fig. 2 show chondrocytes in agarose after seven days in culture above the polymer system. There was a mixture of individual rounded chondrocytes and cell clusters. Extracellular matrix production can be clearly seen in Fig. 2 in between the cells and cellular processes extending into the matrix. There was approximately a three-fold increase in GAG per unit DNA for the polymer system agarose between the seven and 14 day time points. There was no significant difference between the TCP and PEMA/THFMA for GAG production per unit DNA at day seven but there was significantly more produced by the cells above the polymer compared to the TCP at day 14 ($p < 0.03$, students t-test) (Fig. 3). These results are expressed per unit DNA although in fact there was only a slight increase in DNA from days seven to 14 which is indicative of the slow replication rate. Safranin O staining showed a halo of matrix production surrounding the majority of chondrocytes cultured above the polymer system and the TCP although there were a few cells with no matrix staining. Fig. 4 shows Safranin-O staining of chondrocytes cultured for 14 days above the polymer system. Light microscopy showed that chondrocytes adhered to the PEMA/THFMA system by day one (Fig. 5) and were

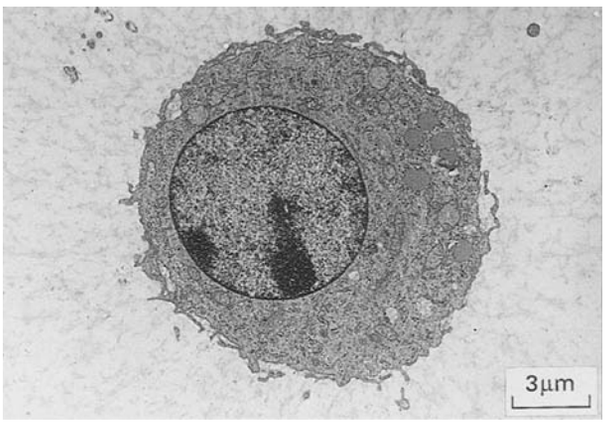

Figure 1 An electron micrograph of a chondrocyte cultured in agarose above the polymer system for seven days.

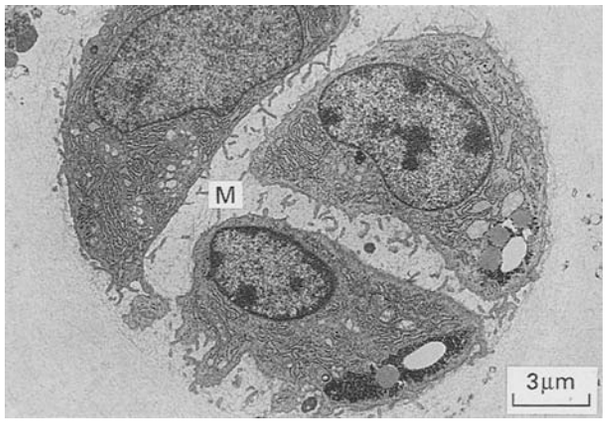

Figure 2 An electron micrograph of a cell cluster cultured in agarose above the polymer system for seven days. Note the cell processes extending into the extracellular matrix (M) in between the cells.

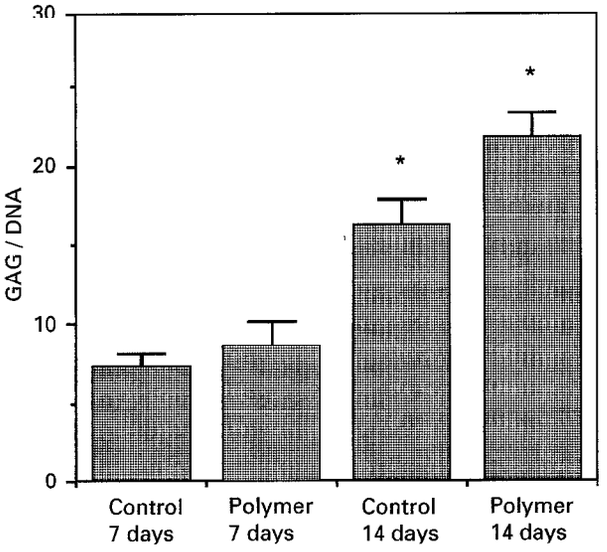

Figure 3 GAG production per unit DNA for chondrocytes cultured in agarose above the PEMA/THFMA system and TCP for seven and 14 days. (Mean +/− SEM, n = 8). The* indicates a significant difference between the TCP and polymer system at day 14 ($p < 0.03$).

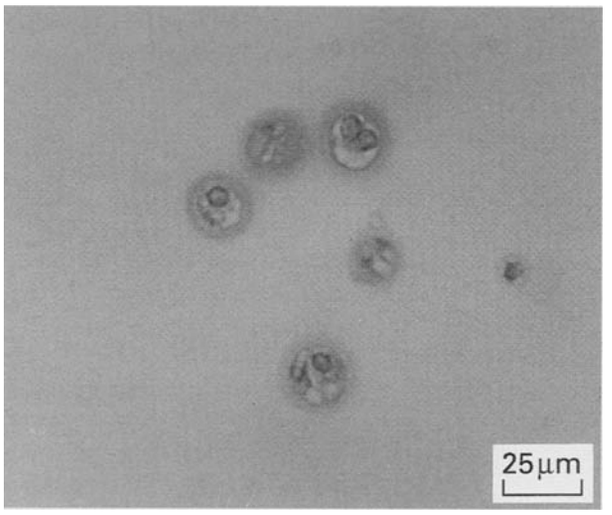

Figure 4 Light micrograph of a histological section of chondrocytes cultured in agarose above the PEMA/THFMA system for 14 days which has been stained with Safranin O and Mayer's haematoxylin.

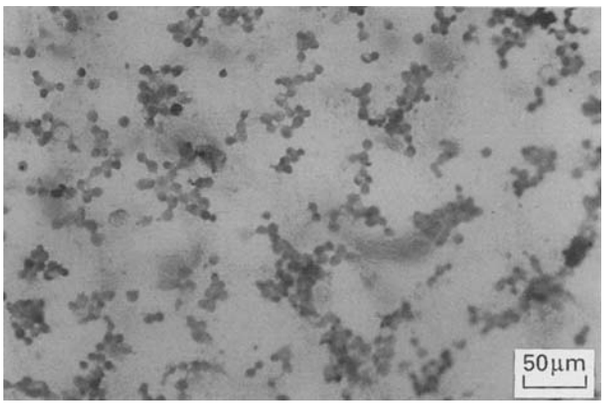

Figure 5 Light micrograph of chondrocytes seeded directly onto the PEMA/THFMA system after one day in culture.

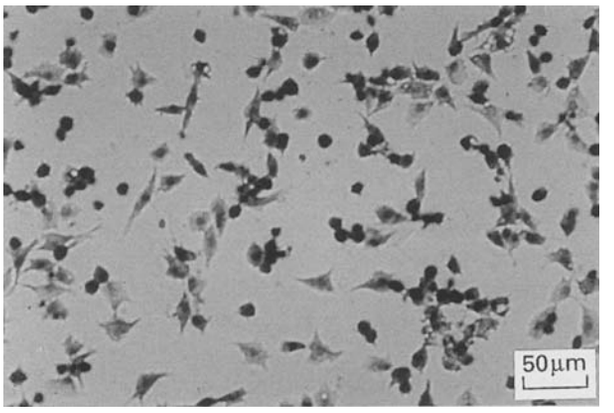

Figure 6 Light micrograph of chondrocytes seeded directly onto Thermanox after one day in culture.

of rounded morphology in contrast to the Thermanox control with many having spread out and become fibroblastic in appearance (Fig. 6).

4. Discussion

The non-degradable polymer system consisting of poly(ethyl methacrylate) (PEMA) and tetrahydrofurfuryl methacrylate (THFMA) has shown potential for cartilage repair in a rabbit model [30–32]. It is a room temperature polymerizing system, has a low exothermic reaction and exhibits low shrinkage, therefore has potential for clinical applications [33, 34]. It has unusual water uptake properties which have been shown to be affected by the osmolarity of the surrounding fluid [unpublished]. We have previously shown by scanning electron microscopy that the polymer system can support chondrocyte growth up to seven days when cells are seeded directly onto the surface [35]. In this work we have shown the PEMA/THFMA system to be biocompatible for bovine chondrocytes supported in agarose and there was no evidence of harmful agents leaching from the polymer system. The agarose cell cultures have shown that the PEMA/THFMA system can support chondrocytes in a viable state with GAG production maintained and a low cell division. The greater GAG production at day 14 for the PEMA/THFMA compared to the TCP

control shows that the polymer may in fact create an advantageous environment for chondrocytes. The Safranin O staining showed that many of the chondrocytes had produced matrix although some showed no matrix production and this is probably because the cells were isolated from full-thickness cartilage and hence contained a mixed population including superficial chondrocytes which produce little matrix [36]. The PEMA/THFMA system supported chondrocyte growth *in vitro* when the cells were seeded directly onto the surface. The cells had a rounded and clustered appearance at day one opposed to well spread with a fibroblastic morphology, as on the Thermanox control. The morphology of chondrocytes has been shown to directly determine the phenotype of the cell, a rounded shape is important to maintain the chondrocyte phenotype and when cells flatten and become fibroblastic in appearance, they dedifferentiate and lose the chondrocytic phenotype [37, 38]. The polymer system we describe here has been shown *in vitro* to maintain chondrocytes in a rounded morphology indicating that their differentiated state is maintained. Chondrocytes maintained on dishes coated with poly-[HEMA] have been shown to remain rounded with a greater rate of GAG production and a lower replication rate compared to cells allowed to flatten [39, 40]. It has been previously suggested that materials which effect chondrocyte phenotype expression *in vitro* may be used as implants to modulate cell expression *in vivo* [41]. The PEMA/THFMA system has been shown *in vitro* to maintain chondrocytes in a differentiated state with enhanced GAG production and this may be important in its success as a repair material *in vivo* [30–32].

5. Conclusions

1. The PEMA/THFMA system has been shown to be biocompatible for bovine chondrocytes.
2. GAG production was greater for the polymer compared to TCP and the chondrocytes had a low cell division.
3. Chondrocytes were maintained in a differentiated state on the surface of the polymer system.

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