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Interactions of chondrocytes with methacrylate copolymers

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Copolymers of poly(ethylmethacrylate) (PEMA) and tetrahydrofurfurylmethacrylate (THFMA) have been shown to exhibit potential as a biomaterial for use in cartilage repair. However, the interactions of chondrocytes with the polymer surface is not well understood. A series of novel methacrylate copolymers containing PEMA, THFMA and hydroxyethylmethacrylate (HEMA) were prepared and the ability of these various copolymers to support chondrocytes attachment *in vitro* has been assessed by the Alamar blue assay for cell number and environmental scanning electron microscopy (ESEM). As the mole fraction of HEMA in PEMA/THFMA/HEMA copolymers increased, chondrocyte attachment to the polymer surface in 24 h decreased. Chondrocytes maintained a rounded morphology and were strongly attached on the THFMA/PEMA polymer surface, but as the mole fraction of HEMA increased the cells present became much smaller with fewer cell to cell interactions. The effect of pre-adsorbing fibronectin on to the polymer surface on cell attachment was assessed both in the presence and absence of serum. Chondrocyte attachment was significantly reduced in serum-free medium. Pre-adsorption of fibronectin on to the copolymer surface substantially increased cell attachment in all cases. In conclusion, chondrocyte attachment and proliferation on these copolymers may be controlled by changes in the polymer surface chemistry and is highly sensitive to the presence of proteins either in the culture media or pre-adsorbed on to the copolymer surface.

1. Introduction

Cell adhesion has been described as the single most important aspect of cell interactions with a biomaterial [1]. This process involves adsorption of proteins on to a biomaterial surface followed by contact and attachment of cells. Surface properties of biomaterials are determined by the chemical and physical properties of the material. These properties influence protein adsorption and configuration which, in turn, affect cell attachment and spreading. Understanding how surface properties influence protein adsorption and configuration and, in turn, cell attachment, is crucial to the development of new biomaterials constructed for specific purposes.

For tissue repair, a material that adsorbs a specific protein in a preferential orientation may be important for tissue integration and cell growth on the surface. It is already well known that cells never see the naked polymer surface because immediately the biomaterial is placed in a biological environment, protein adsorption occurs [2]. Competitive adsorption between the many proteins in biological fluids occurs and it is important which proteins are adsorbed and in which conformation [3]. This, again, is dependent on the surface chemistry of the biomaterial. Chondrocytes have been shown to attach to many extracellular proteins, including the cell adhesion proteins chondronectin, fibronectin and vitronectin [4–6]. Many of these proteins contain an arginine–glycine–aspartic acid (RGD) amino acid sequence which acts as the cell binding site. Therefore, it is not just important that these proteins are present on the biomaterial surface but that the RGD sequence is in the correct orientation for cell attachment.

PEMA/THFMA copolymers have previously been developed as biomaterials for use in cartilage repair [7, 8]. The ability of this polymer system to support chondrocyte growth *in vitro* has also been extensively

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examined, showing that chondrocytes adhere strongly to the polymer surface maintaining their rounded morphology [9, 10]. A similar copolymer system consisting of PEMA/HEMA does not support chondrocyte growth *in vitro* [10].

Although the profound effect of cell–biomaterial interactions on cell attachment, activity, morphology and retention of phenotype is well appreciated and documented, the mechanisms of cell attachment and spreading on biomaterial surfaces are still not well understood.

A series of novel methacrylate copolymers containing PEMA, THFMA and HEMA in different proportions have been prepared, which allows fine control over the biomaterial chemical and physical properties. The effect of these changes on the ability of these copolymers to support chondrocytes *in vitro* has been assessed in terms of cell attachment using the Alamar blue assay for cell number and environmental scanning electron microscopy (ESEM). For chondrocytes to maintain their phenotype it is important that they retain their rounded morphology on the biomaterial surface. Chondrocytes were seeded on to the polymer in either complete medium or serum-free medium to compare cell attachment in the presence and absence of serum proteins. In addition, fibronectin was pre-adsorbed on to the polymer surface to evaluate the influence of a specific cell adhesion protein on chondrocyte attachment.

The aim of these studies was to correlate the effect of copolymer chemistry and protein adsorption with the characteristics of chondrocyte attachment and morphology so as to improve our understanding of the key factors regulating cell–biomaterial interactions and hence aid the development of future biomaterials.

2. Materials and methods

2.1. Polymer preparation

A series of PEMA/THFMA, PEMA/HEMA and PEMA/THFMA/HEMA copolymers were prepared by mixing 5 g PEMA powder (Bonar Polymers Ltd, UK) with a total of 3 ml THFMA and HEMA monomers (Sigma/Aldrich, Gillingham, UK) in various proportions. *N*,*N*, dimethyl-*p*-toluidine (2.5% vol/vol) was included as the activator. The polymer mixtures were either cast directly into a 24-well culture plate or into a polyethylene mold to produce discs of 13 mm diameter and 4 mm thick. The curing time was approximately 10 min. All discs were washed in sterile phosphate-buffered saline solution (SPBS) overnight and sterilized under UV light for 90 min prior to cell culture studies.

2.2. Cell culture

Chondrocytes were isolated from bovine cartilage as previously described [3, 11]. The cell concentration was adjusted to $5 \times 10^5\ \mathrm{ml}^{-1}$ in complete medium (Dulbeccos Modified Eagles medium, Gibco BRL, Paisly, UK, containing 20% foetal calf serum, 2% HEPES, 1% glutamine, 10,000 unit ml^{-1} penicillin/streptomycin and 0.85 mM ascorbic acid) or in serum-free medium (complete medium without 20% foetal calf serum). Aliquots of 1 ml were seeded directly on to the copolymers, thermanox discs or tissue culture plastic (TCP) and incubated at 37 °C in a 5% CO_2 atmosphere.

2.3. Protein adsorption

Bovine fibronectin (Gibco BRL, Paisley, UK) was diluted in sterile phosphate-buffered saline (SPBS) to a concentration of 20 $\mu\mathrm{g\,ml}^{-1}$. Aliquots of 1 ml were added to the copolymers which were previously cast into 24-well culture plates and the plates were incubated at 37 °C for 1 h. The wells were washed twice with SPBS and the chondrocytes immediately seeded on to the polymer. Blank polymer samples were incubated in SPBS that did not contain protein.

2.4. Alamar blue assay

The Alamar blue assay for cell activity was performed after 24 h. The medium was carefully removed from the 24-well plates and the copolymers with adhered cells were washed twice in Earles balanced salt solution (EBSS). Alamar blue (Serotec. Oxford, UK) was diluted 1 : 10 in Hanks balanced salt solution (HBSS). Of this solution 1 ml was added to each well and incubated at 37 °C for 90 min. A 100 µl aliquot was transferred to a 96-well plate and the fluorescence measured at excitation wavelength of 530 nm and emission 590 nm, using a Perspective Biosystems Cytofluor 2300. This method for assessing chondrocyte attachment has been previously evaluated [12]. The results are reported as a percentage of the measured fluorescence for chondrocytes seeded on TCP in complete medium.

2.5. Morphological analysis with ESEM

After 5 d incubation, the medium was removed and the polymer discs washed twice in SPBS. The cells were fixed in 1.5% gluteraldehyde in 0.1 M sodium cacodylate buffer for 4 h and stored in SPBS prior to analysis. These samples were analyzed using a Philips XL30 environmental scanning electron microscope equipped with a field emission gun (ESEM-FEG) in wet mode to retain the cells in a fully hydrated condition.

3. Results

3.1. Effect of polymer chemistry on cell attachment

The composition of the PEMA/THFMA/HEMA copolymers investigated in this study is represented in Fig. 1 as the ratio of THFMA/HEMA monomer components. Chondrocyte attachment to these polymers was evaluated using the Alamar blue assay after 24 h. When chondrocytes were seeded in complete medium (containing 20% serum) cell attachment was significantly greater on the copolymer containing PEMA/THFMA than on any of the copolymers containing HEMA (Fig. 1). As the proportion of HEMA

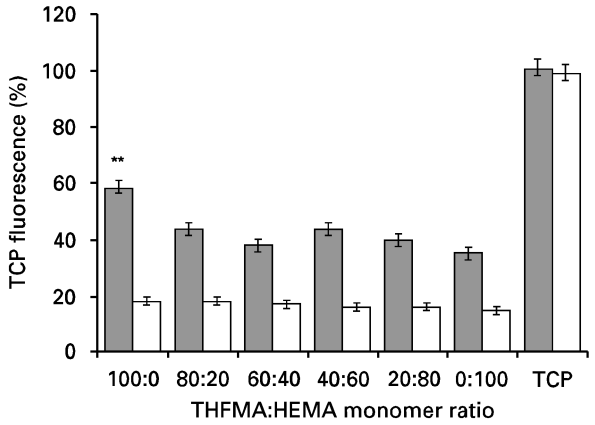

Figure 1 Graph showing chondrocyte attachment to PEMA copolymers prepared with different THFMA:HEMA monomer ratios. The cells were seeded in (■) complete medium. and (□) serum-free medium and cultured for 24 h. The Alamar blue assay was employed and the results are represented as per cent fluorescence units of tissue culture plastic with complete medium. Each bar shows the ±standard error of means for four replicates. (**) A significant difference to the other samples ($P < 0.01$). There is a significant difference between all complete medium (■) and serum-free medium (□) samples of $P < 0.001$ except the TCP.

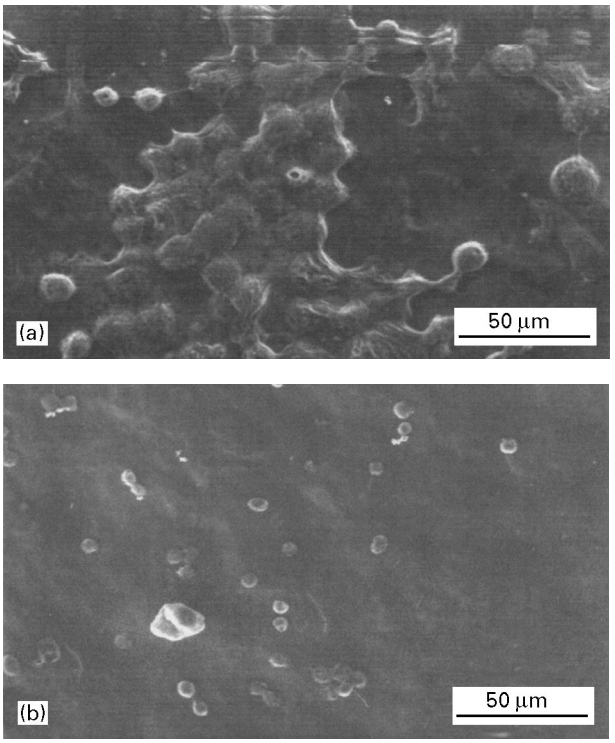

Figure 2 Environmental scanning electron microscope image of a representative sample of chondrocytes on (a) PEMA/THFMA, and (b) PEMA/HEMA.

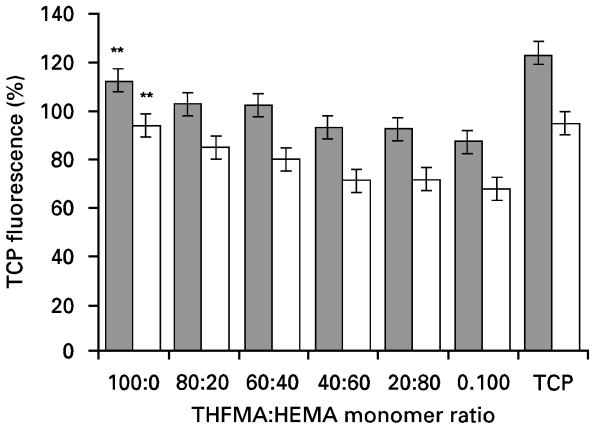

Figure 3 Graph showing chondrocyte attachment to PEMA copolymers prepared with different THFMA:HEMA monomer ratios. Fibronectin was pre-adsorbed on to the polymer surface prior to the addition of cells. The cells were seeded in (■) complete medium and (□) serum-free medium, and cultured for 24 h. The Alamar blue assay was employed and the results are represented as per cent fluorescence units of tissue culture plastic, without fibronectin pre-adsorbed, with complete medium. Each bar shows the ±standard error of means for four replicates. (**) A significant difference to the other samples ($P < 0.01$). There is a significant difference between all complete medium (■) and serum-free medium (□) samples of $P < 0.01$.

increased there was a clear and general trend of decreasing chondrocyte attachment, although this was not statistically significant. In serum-free medium, cell attachment was much lower on all the copolymers evaluated (Fig. 1) and there was no significant difference in attachment between the different polymer samples.

3.2. Effect of polymer chemistry on chondrocyte morphology

A representative sample of chondrocytes attached to the PEMA/THFMA copolymer and imaged using ESEM after 5 d is shown in Fig. 2. It was observed that the cells were beginning to cover the polymer surface with processes extending on to the surface. These cells exhibit a rounded morphology, a favorable indicator for retention of the chondrogenic phenotype. In contrast, chondrocytes attached to the PEMA/HEMA copolymer after 5 d (Fig. 2b), although still rounded, were much smaller, isolated cells scattered across the surface.

3.3. Effect of pre-adsorbed fibronectin on chondrocyte attachment

It is known that serum proteins adsorbed to biomaterial surfaces play an important role in cell attachment. The effect on chondrocyte attachment of pre-adsorbing fibronectin on to the copolymer surface was measured as before, for cells seeded in either complete medium or serum-free medium. Fig. 3 shows that for all copolymers tested the cell attachment was significantly greater ($P < 0.001$) in complete media than in serum-free medium. When fibronectin was pre-adsorbed on to copolymer samples, cell attachment was significantly greater ($P < 0.001$) than when fibronectin was not adsorbed (compare Fig. 1 with Fig. 3). The trend of decreasing cell attachment with increasing HEMA content was still apparent when fibronectin was pre-adsorbed on to the copolymer surface.

4. Discussion

Varying the composition of PEMA/THFMA/HEMA copolymers results in differences in chondrocyte attachment and morphology. However, these

differences are only apparent when serum proteins are present in the culture media. We can therefore speculate that the differences in cell attachment are not directly due to the effect of the surface chemistry on the cells but rather stem indirectly from the altered ability of the different polymers to adsorb serum proteins. It has previously been shown that fibronectin and vitronectin adsorption is enhanced on PEMA/THFMA polymers relative to PEMA/HEMA copolymers [13]. Therefore it is possible that the increase in cell attachment is due to an increase in the amount of adsorbed protein. Different surface chemistries may also result in serum proteins being adsorbed in different conformations. Changes in the relative amounts of adsorbed protein, the type of protein adsorbed and conformational differences may give rise to the presentation of peptide sequences which are more accessible for cell attachment on the PEMA/THFMA copolymers relative to the PEMA/HEMA polymer.

Chondrocytes attached to the PEMA/THFMA polymer maintain their rounded morphology. In contrast, as the mole fraction of HEMA increases, cell attachment decreases and, by a monomer ratio of 30:70 (THFMA:HEMA), the cells were smaller and scattered across the surface suggesting that they were not proliferating to the same extent. This difference in morphology cannot be explained simply by differences in the amount of protein adsorbed and may be directly due to altered chemical or physical properties of the HEMA containing copolymers.

Protein adsorption on to biomaterial surfaces is an important prerequisite for cell attachment and, in this present study, surface modification with pre-adsorbed fibronectin has been shown to result in a great improvement in cell attachment with a variety of copolymers. A similar trend of decreasing cell attachment with increasing mole fraction of HEMA was observed when fibronectin was pre-adsorbed on to the polymer surface. This is possibly due to less fibronectin adsorbing on to the HEMA copolymers. However, all samples showed a significant increase in cell attachment compared to the samples without pre-adsorbed fibronectin. These findings indicate that PEMA/THFMA/HEMA copolymers are capable of adsorbing fibronectin from a SPBS solution, subsequently improving cell adhesion. Fibronectin in the serum may not adsorb as efficiently due to competitive adsorption from other proteins or the fibronectin adsorbed is not in the correct orientation for cell attachment. There were many more cells attached to all copolymer samples in serum-free medium after pre-adsorption of fibronectin compared to blank polymers in complete medium. This confirms that fibronectin is important for cell attachment and that it functions more efficiently when pre-adsorbed to surfaces rather than under conditions of competitive adsorption with other serum proteins. Maximum cell attachment was observed when fibronectin-coated copolymers were incubated in complete medium which suggests that although fibronectin is important, other serum proteins are also required for optimizing cell attachment. While fibronectin is the best protein for cell attachment if it is pre-adsorbed and does not have to compete with other proteins for adsorption sites vitronectin is considered better at competing with other serum proteins [12]. The effect of vitronectin on chondrocyte attachment will be monitored in future work.

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