

# Surface-functionalised materials for biomedical applications

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## ABSTRACT

Electrospun fibrous matrices prepared from methacrylate-based copolymers are investigated as a tool for retinal pigment epithelium (RPE) transplantation in the treatment of degenerative retinal diseases. Human RPE cells were used to probe the cell-surface interactions on these copolymer matrices. For the first time, simple changes in chemical functionality have been found to induce gel formation of these methacrylate backbone copolymers *in vitro*. This effect is shown to significantly improve RPE cell adhesion and survival.

**Keywords:** AMD, MMA, PLGA, electrospinning, ARPE-19

## 1. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world [1]. Damage to the retinal pigment epithelium (RPE), which in turn leads to photoreceptor death, is characteristic of debilitating degenerative retinal disease like AMD [2]. As a consequence, the RPE and its supportive structure known as Bruch's membrane (BM) have become targets for tissue engineering researchers. Transplantation of a synthetic BM with a healthy RPE has the potential to restore vision by supporting photoreceptor function. RPE cells are anchorage-dependent; and it is therefore imperative that a supportive biomaterial be designed to promote cellular attachment. Various natural and artificial matrices have been investigated for this purpose [3], however little is known about chemically altering materials to specifically improve adhesion of RPE cells [4].

Engineering a material capable of mimicking the extracellular environment presents a great challenge for materials scientists. Different approaches have been used for RPE transplantation including scaffolds based on ECM proteins [5] and natural tissues [6]. Matrices derived from natural sources, however, have inherent problems with respect to high cost, purity and lack of availability.

The limitations of using naturally derived substrates have led many groups to pursue the use of synthetic polymers as scaffolds for RPE cell delivery. Whilst biodegradable polymers [7] have proven popular for this application, non-biodegradable polymers have also been employed. Surface modification of various materials has been utilised to improve their biocompatibility [8]. These modifications often target improvements in cellular

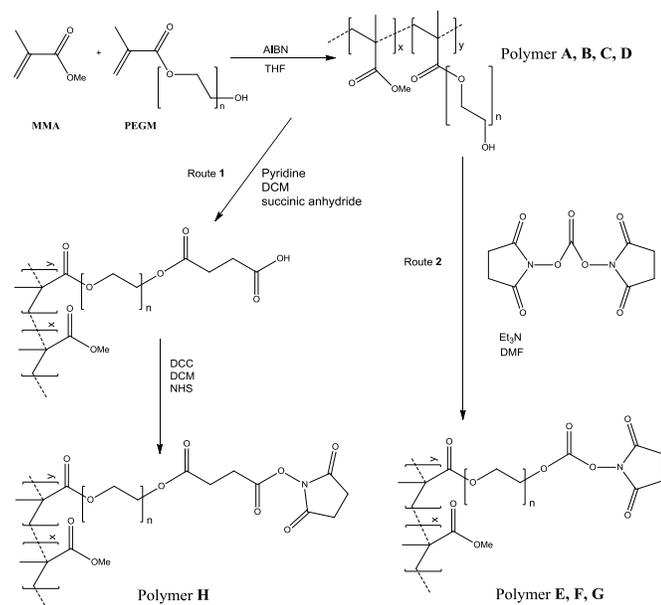
adhesion through the use of ECM proteins or short chain peptides which have recognised cell binding domains [9]. Using peptides and proteins to regulate cell adhesion have drawbacks such as high cost and purification difficulties [10].

## 2. RESULTS AND DISCUSSION

Copolymers of methyl methacrylate (MMA) and poly(ethylene glycol) methacrylate (PEGM) were successfully synthesised and further functionalised as outlined in Scheme 1. Succinimidyl groups are widely used in the literature to facilitate protein and peptide coupling to polymer substrates [9]. Using various blend ratios, either a succinimidyl carbonate (Polymers **E**, **F** and **G**) or a succinimidyl ester (Polymer **H**) group was introduced (table 1). After the polymers were electrospun into fibers, cell adhesion was assessed using these copolymer mats. The change in functionalization and affect on cell adhesion have not previously been attempted for these methacrylate copolymer systems. NMR and IR spectroscopy were used to confirm the successful removal of any residual monomer and the addition of groups to the poly(ethylene glycol) (PEG) chain terminus. The appearance of peaks in the proton NMR spectrum at ca.  $\delta$ 2.8ppm confirmed the addition of protons from a succinimidyl group. This is further demonstrated by the appearance of a new carbonyl peak in the IR spectrum indicative of the presence of a cyclic imide. In addition to this, the appearance of a peak around  $\delta$ 4.25ppm for protons adjacent to a new ester or carbonate linkage confirms coupling of a succinimidyl group to the end of the PEG chain.

Contact angle experiments were used to determine the relative hydrophilicity of the various copolymers (Table 2). Despite having a similar contact angle, Polymer **D** (containing HEMA ( $n=1$ ) units) showed increased cell adhesion properties compared to Polymer **C** (PEG (Av MW ~526) containing copolymer). This suggests that the presence of the PEG chain exerts an influence on cell adhesion beyond that of simply increasing hydrophilicity within the copolymer. Conversion of the PEG terminus alcohol group into either a succinimidyl carbonate or an ester resulted in an increase in hydrophobicity. This effect was more pronounced in the copolymers containing longer PEG chains (Table 2). It was noted that when the contact angle measurement was obtained for polymer **F** and **G** using the electrospun fibers that full absorption of the water droplet into the mat occurred after 10 seconds. Although the

contact angles for these polymers were greater (more hydrophobic) when simply spun onto a glass surface, when tested as the fibers they were far more hydrophilic and effectively had a contact angle of zero. This might be expected as these polymers have a greater number of PEG groups present and the addition of a succinimidyl group does not make the fibers more hydrophobic as initially observed.



Scheme 1: Preparation of polymers A-H

It was noted that when the succinimidyl carbonate polymers were left in solution, an insoluble hydrogel was produced. Further to this, addition of an excess of base to a solution of purified succinimidyl carbonate or ester functionalized polymer also gave an insoluble gel. Copolymers with a higher percentage of PEG groups present produced a hydrogel faster than polymers with a greater percentage MMA. This effect has not previously been observed in the literature with these polymers. Some initial experiments showed us that the succinimidyl carbonate group can ring open to form a crosslink with any unreacted PEG groups which still have the OH end group present [11].

The synthesized copolymers (Table 1) were used to prepare a fibrous matrix using an electrospinning technique [12]. Scanning electron microscopy was used to confirm the size and morphology of these fibers (Fig. 1). All of the matrices produced had an average fiber diameter of less than 10 $\mu$ m (Table 2). It was found that matrices of smaller fibers gave handling advantages for cell seeding compared to larger diameter fibers. Material handling properties are an important factor when considering the future surgical applications of these matrices. Unmodified polymers tended to have a slightly larger average fiber diameter than that of their modified counterparts. Electrospinning of Polymer **D** resulted in the formation of ribbon-like fibers with necking observed in the middle of each ribbon (Fig. 1C). The

average fiber diameter for polymer **D** was significantly larger ( $p < 0.001$ ) than of the other fibers tested.

Polymer	% MMA	% PEGM	PEGM Av MW	End group
A	60	40	~360	OH
B	40	60	~360	OH
C	50	50	~526	OH
D	50	50	62 (HEMA)	OH
E	60	40	~360	Succinimidyl carbonate (Route 2)
F	40	60	~360	Succinimidyl carbonate (Route 2)
G	50	50	~526	Succinimidyl carbonate (Route 2)
H	60	40	~360	Succinimidyl ester (Route 1)

Table 1: Monomer feed ratios for the different co-polymers

This collapse of the electrospinning jet into a ribbon is a major contributor to the wider average diameter of these fibers. In addition to individual fiber diameter, average scaffold porosity was also assessed (Table 2). The least porous scaffold was produced using polymer **E**, while the most porous scaffold produced was manufactured using polymer **H**. Scaffold porosity did not appear to correlate with fiber diameter.

Polymer	Fiber diameter ( $\mu$ m) (n = 15) <sup>a</sup>	Scaffold porosity (%) (n = 10) <sup>b</sup>	Contact angle ( $^{\circ}$ ) (n = 3) <sup>c</sup>
A	2.05 $\pm$ 0.27 +	34.82 $\pm$ 2.48	59.7 $\pm$ 2.9
C	2.24 $\pm$ 0.22 ++	30.98 $\pm$ 1.89	57.0 $\pm$ 0.7
D	9.82 $\pm$ 0.73 ***	31.88 $\pm$ 3.30	58.6 $\pm$ 0.4
E	1.80 $\pm$ 0.11	22.54 $\pm$ 1.34	67.8 $\pm$ 0.4
F	1.36 $\pm$ 0.10	33.17 $\pm$ 1.93	74.6 $\pm$ 0.6
G	1.44 $\pm$ 0.11	29.90 $\pm$ 4.47	77.5 $\pm$ 1.2
H	1.34 $\pm$ 0.20	35.18 $\pm$ 3.13	67.1 $\pm$ 0.8

Table 2: Table to show mean fiber diameter, scaffold porosity and contact angle for different copolymers

<sup>a, c</sup> Values expressed as mean  $\pm$  sem. Statistical analysis by Student's t-test. Statistical difference \*\*\* $p < 0.001$  compared to all other fibers tested. Fibers of polymer A (+  $p < 0.05$ ) and C (++  $p < 0.01$ ) were statistically different compared to fibers of polymers **F**, **G** and **H**. <sup>b</sup>Statistical analysis by ANOVA followed by post-hoc comparison.

Differences observed in scaffold porosity did not reach statistical significance. As native BM is a diffusion barrier,

porosity is an important property to measure in these systems. We are currently investigating the diffusion and mechanical characteristics of these fibrous matrices in relation to human BM.

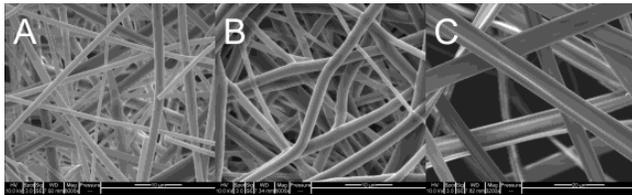


Fig.1: Electron micrographs showing fibers of (A) Polymer A, (B) Polymer E, and (C) Polymer D. Scale bar 10µm (A,B), 20µm (C).

Polymer B was found to dissolve at room temperature in water and therefore could not be used for cell testing. This is most likely due to the increase in PEG in this polymer resulting in increased hydrophilicity. Human RPE cells were successfully seeded onto the surface of all of the fibrous polymer networks prepared in this study. Growth of a cellular monolayer on the surface of the fibers was quantified by nuclear labeling (Fig. 2). Minimal cell attachment was observed on the polymer fibers containing alcohol-terminated PEG chains (polymers A and C). By day 15, the percentage of cell adhesion had decreased on fibers of polymers A and C from approximately 3% to  $0.4\% \pm 0.1$  and  $1.3\% \pm 0.3$  respectively. On all other fibers tested percentage cell area increased with time *in vitro*. The most significant increase in cell area ( $38\% \pm 5.9$ ,  $p < 0.001$ ), was noted with fibers of polymer G. Less cell attachment however, was observed overall on fibers of this polymer in comparison with other succinimidyl functionalised polymers. This is likely to be a result of the presence of the longer PEG chain increasing the hydrophilicity of the polymer and therefore decreasing cell adhesion.

Cells were noted to have a characteristic epithelial shape with defined cell borders when observed on fibers by SEM. Apical microvilli were also present suggesting appropriate polarisations and functionality. Overall, we observe that when the PEG-containing polymers were functionalised with a succinimidyl group either using an ester or carbonate linkage, cell attachment was significantly increased compared to polymers containing alcohol terminated PEG. Further to this, polymer fibers with this functionalisation were observed to form a gel *in vitro* and as a consequence became insoluble. We propose that the polymer chains are undergoing a crosslinking process resulting in a change in physical properties. This provides a possible reason for increased adhesion and proliferation of cells on these fibrous surfaces.

Apoptotic cell death on copolymer fibers was quantified by TUNEL assay. There was minimal cell death noted throughout the investigation period for fibers prepared from polymers D, E, F, G and H. The proportion of TUNEL positive cells was not significantly different when compared with the negative control. Percentage cell

death was observed to decrease on fibers of polymer F and G with time *in vitro*, while on fibers of polymer A ( $37\% \pm 7.5$ ) and C ( $50\% \pm 10$ ) there was a significant increase in cell death from day 5 to day 15 *in vitro*.

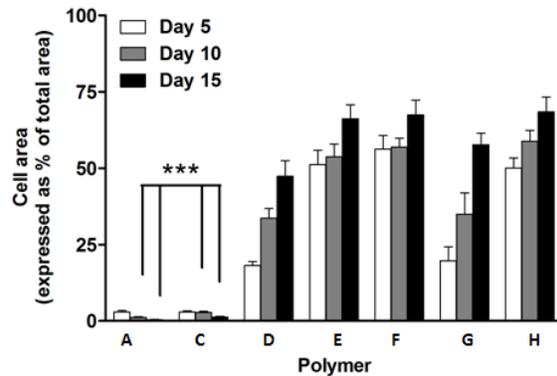


Fig. 2: Bar graph depicting percentage ARPE-19 cell area, assessed by 60 nuclear labeling. Results represent mean  $\pm$  sem (n= 10). Values are grouped according to polymer and by time *in vitro*. Statistical analysis by ANOVA followed by Tukey-Kramer multiple comparison test. At days 10 and 15 there was a significant difference (\*\*\*)  $p < 0.001$  in percentage cell area on polymers A and C compared to polymers D, E, F, G and H.

Apoptotic cell death on fibers of polymers A and C did not significantly differ when compared to positive control. As RPE cells are anchorage dependent, the lack of adhesion observed on fibers of polymer A and C may explain the high levels of cell death observed on these.

It is important that tissue engineering scaffolds do not have any cytotoxic effects on the tissues they are intended to aid. To investigate this for our polymer systems, an MTT assay was used as an indirect measure of cytotoxicity. The results of this assay show a slight decrease in cell viability compared to the control (cells incubated with normal tissue culture medium). This effect however, did not reach significance with any of the polymers tested. The lowest cell viability was noted with fibers of polymers A and C ( $90.5\% \pm 4.1$  and  $88.1\% \pm 3.6$ ).

### 3. CONCLUSION

Overall, these results show that the methacrylate copolymers with an alcohol terminated PEG chain gave significantly lower cellular adhesion and survival over succinimidyl capped polymers. When the functional group on the end of the PEG chain was changed to either a succinimidyl carbonate or succinimidyl ester, the polymer formed a crosslinked gel *in vitro*. Fibers of these polymers showed significantly improved cell adhesion and survival properties. There have been studies of photocrosslinkable polymers formulated into fibers for tissue engineering applications [13]. However, investigation of this type of succinimidyl crosslink polymer material has not previously

been reported in the literature and shows considerable promise for further study. Cellular scaffolds made from this material have the potential to aid RPE transplantation in patients with retinal degenerative diseases. Furthermore, a polymer that can crosslink into a gel after being manufactured into a fibrous matrix has potential use for other tissue engineering applications in many tissue types and diseases.

## 4. MATERIALS AND METHODS

### 4.1 Synthesis of a co-polymers of methyl methacrylate and poly(ethylene glycol) methacrylate, Polymers A, B, C and D

To tetrahydrofuran (THF) (150 ml per 12g total polymer) were added methyl methacrylate (MMA) (wt %) and poly(ethylene glycol) methacrylate (PEGM) (wt %). The solution was degassed ( $N_2$ ) for 20 min. Azobisisobutyronitrile (AIBN) (Acros Organics, Geel, Belgium) (0.00058 mol, 0.096 g) was then added before the solution was refluxed overnight under  $N_2$ . The resulting solution was purified twice by precipitation in diethyl ether (3 x 200ml) yielding the final solid. FT-IR  $\nu_{max}/cm^{-1}$  2991 and 2948 (CH), 1721 (CO).  $^1H$  NMR: $\delta$ ppm (400MHz,  $CDCl_3$ ) 0.84 (br s), 1.01 (br s), 1.81 (br s), 1.86 (br s), 3.59 (br s), 3.66 (br s), 4.11 (br s).  $^{13}C\{^1H\}$  NMR: $\delta$ ppm (100 MHz,  $CDCl_3$ ) 16.5, 18.8, 44.6, 44.9, 51.8, 54.4, 61.7, 63.0, 67.9, 68.6, 70.6, 72.6, 177.0, 177.8

### 4.2 Synthesis of P(MMA-co-PEGM-succinimidyl carbonate)– Polymers E,F and G

To dichloromethane (DCM) (100ml) was added either Polymer A, B or C (7g), disuccinimidyl carbonate (DSC) (Acros Organics) (0.0098mol, 2g) and triethylamine (2ml). The mixture was left to stir overnight and the product was then precipitated in diethyl ether (200ml) yielding a white solid. This was redissolved in DCM and reprecipitated in diethyl ether (3 x 100ml) to give a white solid which was then dissolved in chloroform, washed with HCl aq (2M) and dried over  $MgSO_4$ . The solvent was evaporated *in vacuo*. FTIR  $\nu_{max}/cm^{-1}$  2993 and 2950 (CH), 1790 (CO-N cyclic imide), 1722s (CO).  $^1H$  NMR: $\delta$ ppm (400MHz,  $CDCl_3$ ) 0.85 (br s), 1.03 (br s), 1.62 (br s), 1.82 (br s), 2.86 (br s), 3.61 (br s), 3.67 (br s), 4.12 (br s), 4.48 (br s).  $^{13}C\{^1H\}$  NMR: $\delta$ ppm (100 MHz,  $CDCl_3$ ) 16.5, 18.7, 25.6, 44.6, 44.9, 80 51.8, 54.4, 68.0, 68.3, 70.6, 177.8, 178.1.

### 4.3 Synthesis of P(MMA-co-PEGM-succinimidyl ester)– Polymer H

To DCM (100ml) was added Polymer A (8g) and succinic anhydride (Acros Organics) (0.03mol, 3g). The resulting solution was degassed with  $N_2$  for 20 min. Pyridine (2ml) was then added before the solution was refluxed for 2 hrs. The solvent volume was reduced *in vacuo* and the solution was precipitated in diethyl ether (200ml). The resulting solid was dissolved in chloroform (100ml) and washed with an equal volume of HCl aq (2M). The organic layer was separated and precipitated in diethyl ether (200ml) to give the polymer as a solid (5.35g).

To DCM (100ml) was added carboxylated Polymer (5g) and N-hydroysuccinimide (Acros Organics) (NHS) (0.01mol, 1.2g). Separately, N,N'-dicyclohexyl carbodiimide (DCC) (Acros Organics) (0.006mol, 1.2g) was dissolved in DCM (50ml). The DCC solution was added dropwise to the polymer/NHS solution. The reaction mixture was stirred overnight at room temperature. The resulting suspension was filtered through cotton wool to remove dicyclohexyl urea (DCU) and then precipitated in diethyl ether (200ml). The resulting solid formed was dissolved in chloroform (50ml) and the solvent was removed *in vacuo* yielding a transparent solid (4.5g). FTIR  $\nu_{max}/cm^{-1}$  2949 and 2993 (CH), 1784 (CO-N cyclic imide), 1722s (CO).  $^1H$  NMR: $\delta$ ppm (400MHz,  $CDCl_3$ ) 0.85 (br s), 1.02 (br s), 1.81 (br s), 2.84 (m), 2.96 (m), 3.59 (br s), 3.64 (br s), 4.11 (br s), 4.28 (br s).  $^{13}C\{^1H\}$  NMR: $\delta$ ppm (100 MHz,  $CDCl_3$ ) 16.5, 18.7, 25.4, 25.6, 26.2, 28.7, 44.5, 44.9, 51.8, 54.3, 61.7, 64.2, 68.5, 68.9, 70.6, 72.6, 72.1, 169.0, 171.0, 177.0, 177.8, 178.1

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