Biocompatibility of Poly(2-alkyl-2-oxazoline) Brush Surfaces for Adherent Lung Cell Lines

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Abstract

Development of synthetic surfaces that are highly reproducible and biocompatible for *in vitro* cell culture offers potential for development of improved models for studies of cellular physiology and pathology. They may also be useful in tissue engineering by removal of the need for biologically-derived components such as extracellular matrix proteins. We synthesised four types of 2-alkyl-2-oxazoline polymers ranging from the hydrophilic poly(2-methyl-2-oxazoline) to the hydrophobic poly(2-*n*-butyl-2-oxazoline). The polymers were terminated using amine-functionalised glass coverslips, enabling the synthetic procedure to be reproducible and scaleable. The polymer-coated glass slides were tested for biocompatibility using human epithelial (16HBE 14o-) and fibroblastic (MRC5) cell lines. Differences in adhesion and motility of the two cell types was observed, with the poly(2-isopropyl-2-oxazoline) polymer equally supporting the growth of both cell types, whereas poly(2-*n*-butyl-2-oxazoline) showed selectivity for fibroblast growth. In summary, 2-alkyl-2-oxazoline polymers may be a useful tool for building *in vitro* model cell culture models with preferential adhesion of specific cell types.

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1. Introduction

Cell culture systems are important for investigating fundamental processes and mechanisms controlling normal cellular physiology, as well as alterations in disease. They also have applications in tissue engineering where regulatory demands place a significant burden on quality control, reproducibility and control over raw materials. Currently tissue culture plasticware is fabricated from polystyrene; this provides a hydrophobic surface that does not support growth of adherent cells. Thus, these hydrophobic surfaces are used for suspension cultures and in the creation of 3D spheroids [1]. However, the polystyrene surfaces can be plasma treated to enhance their hydrophilicity, thereby increasing protein absorption on to the surface from serum components contained within the culture medium, allowing cell adhesion via integrin binding [2]. To further improve cell adhesion, extracellular matrix (ECM) proteins, for example collagen, can be used to pre-coat plasticware, providing more integrin binding sites. However, there are several potential problems associated with use of ECM proteins which are derived from biological sources since they may contain adventitious agents and can show considerable batch variation. In addition, they do not always provide an even coating. For these reasons, alternative non-biological modifications are being investigated to improve cell specific adhesion to tissue culture plastic or other substrates.

Poly(2-alkyl-2-oxazoline)s are an interesting group of polymers particularly for biological applications. Firstly, poly(2-alkyl-2-oxazoline)s have a low toxicity [3-5], indeed ethyl poly(2-oxazoline)s have already been approved by the FDA as indirect food contact agents [6]. These two polymers also display a ‘stealth’ behaviour, which means they show reduced interactions with immune system proteins [7-8]. For example, addition of 2-methyl-2-oxazoline (MeOx) and 2-ethyl-2-oxazoline (EtOx) polymers to cultures of a mouse

macrophage cell line did not affect cell viability or influence their capacity for immune activation [9].

Although poly(2-oxazoline)s were discovered in the 1960s [10-12], they had almost been forgotten up to the 1990s mainly because of the long reaction times required and limited application possibilities. With the discovery of the potential biological relevance of these polymers and the advent of microwave assisted polymerisation dramatically reducing reaction times, the field has been re-invigorated [13-14]. Several biological applications have already been studied involving these polymers [15], including drug delivery using micelles [16] or conjugation to a drug or protein [17]. Co-polymers have been investigated for the creation of antimicrobial surfaces [18], to block protein and cell adhesion for coating implant surfaces [19-20] and as a hydrogel for tissue engineering [21].

Poly(2-oxazoline)s are well suited for surface functionalization as they have a low polydispersity index due to the monomeric addition achieved via a living cationic ring opening polymerisation (CROP). The side chain can be easily modified with amines [22] or carboxylic acids [23] thereby allowing for further enhancement of the surface via peptide conjugation or other functionalization. For cell adhesive surfaces it has been shown that attachment of poly(2-ethyl-2-oxazoline) (PEtOx) to glass allows the growth of human umbilical vein endothelial cells (HUVECs) and primary rat and sheep fibrocytes [24]; HUVECs can also adhere and spread on fibronectin-coated PEtOx and poly(2-methyl-2-oxazoline) PMeOx attached to glass [19]. Very little else is known about the biocompatibility of related oxazoline polymers, in particular their utility for supporting cell growth or their selectivity for different cell types. In this study we have investigated four oxazoline polymers with a range of hydrophobicities (from most hydrophilic to most hydrophobic): poly(2-

methyl-2-oxazoline) (PMeOx), poly(2-ethyl-2-oxazoline) (PEtOx), poly(2-isopropyl-2-oxazoline) (P*i*PrOx) and poly(2-*n*-butyl-2-oxazoline) (P*n*BuOx). We explored their biocompatibility and potential to provide a surface that could select for a specific cell type out of a mixture of cells.

2. Materials and Methods

2.1. Materials

All chemicals for the polymerisation and surface functionalisation were purchased from Sigma-Aldrich (Poole, UK) and used without further purification unless specified. All cell culture products were obtained from Life Technologies (Paisley, UK) unless otherwise specified.

2.2. XPS measurements

XPS measurements were carried out using a Thermo Fisher ME17 Thetraprobe XPS system with a monochromatic A1 X-ray source, set to a 400µm spot size. The scan count was 5 scans for the overview spectra and 20 scans for the elemental binding energy spectra. Deconvolution of the spectra was performed in Excel by fitting the data to multiple Gaussian bands, reducing the residual square to a minimum. To correct for any charging, the C-C bonding peak was used as a reference peak of binding energy 285.0 eV [25]. Contact angle goniometry was performed using a Kruss DSA 100 drop shape analyser running SMARTDROP contact angle software on a Windows PC. The most suitable fitting method for the drop was used depending on how hydrophobic or hydrophilic the substrate was with a drop volume of 1µL of ultrapure (18 MΩ·cm) H_2O. IR spectra were collected with a Nicolet 380 FT-IR spectrometer with a SmartOrbit golden gate attenuated total reflection (ATR) attachment. Microwave reactions were performed using a CEM discover microwave reactor

equipped with an autoloader. This was connected to a Windows PC running CEM discover software.

2.3. Synthesis of 2-alkyl-2-oxazolines

Isopropyl or butyl nitrile (2.22M), ethanolamine (163ml, 2.66M) and zinc acetate (24g, 0.111M) were stirred for 20hrs at 130°C. The crude yellow oil was then distilled to yield the monomer as a colourless oil. 2-isopropyl-2-oxazoline - distilled at 50°C, (70% yield). - ^{1}H NMR (300Mhz, $CDCl_3$, δ/ppm): 1.19 (d, *J*=6.9 Hz, 6H), 2.56 (spt, *J*=6.9 Hz, 1H), 3.81 (t, *J*=9.7 Hz, 2H), 4.22 (t, *J*=9.3 Hz, 2H). ^{13}C NMR (300Mhz, $CDCl_3$, δ/ppm): 19.7, 28.1, 54.3, 67.2, 172.6. IR (ν/cm^{-1}): 2971-2880 (C-H), 1663 (N=C), 1142 (C-O). 2-Butyl-2-oxazoline – distilled twice at 70°C, (40% yield). - ^{1}H NMR (300Mhz, $CDCl_3$, δ/ppm): 0.91 (t, *J*=7.33 Hz, 3H), 1.36 (dq, *J*=15.03, 7.28 Hz, 2H), 1.60 (dt, *J*=15.41, 7.45 Hz, 2H), 2.26 (t, *J*=7.58 Hz, 2H), 3.80 (t, *J*=9.60 Hz, 2H), 4.20 (t, *J*=9.35 Hz, 2H). ^{13}C NMR (300Mhz, $CDCl_3$, δ/ppm): 13.7, 22.3, 27.6, 28.0, 54.3, 67.0, 168.6. IR (ν/cm^{-1}): 2971-2880 (C-H), 1663 (N=C), 1142 (C-O).

2.4. Amine coating of glass coverslips

Glass coverslips were cut to size (ca. 1 x 2 cm) using a diamond-tipped pen and placed in a solution of concentrated sulphuric acid:hydrogen peroxide (3:1 v/v; CAUTION: reacts vigorously with any organic compound, keep well away from sources of organic chemicals). After 1h, the slides were washed with copious amounts of ultrapure H_2O followed by ethanol. Each slide was then immersed in a solution of (3-aminopropyl)triethoxysilane (38µl) in freshly distilled ethanol (5ml) for 1 h. The slides were removed and washed with copious amounts of ethanol before being heated to 80°C for a further 1h.

2.5. 2-Alkyl-2-oxazoline polymerisation

For each batch, a volume of polymer stock solution was used to provide the same number of moles of monomer in each reaction of 2-methyl-2-oxazoline (22ml), 2-ethyl-2-oxazoline (26.22ml), 2-isopropyl-2-oxazoline (31.07ml) and 2-butyl-2-oxazoline (35.91ml). Briefly, acetonitrile (44ml), 2-alkyl-2-oxazoline (259.81mmol) and methyl *p*-toluenesulfonate (0.261ml, 1.73mmol) were thoroughly mixed and then split into aliquots of 3ml in each of 24 microwave vials. Each vial was subjected to microwave irradiation for 15 min (135°C). The polymer mixtures were then used immediately for coating glass coverslips. For each polymer batch an aliquot (1ml) was removed and sat. KOH in methanol was added (100µl) and the sample was stirred overnight at 60°C. Subsequent precipitation in ice cold diethyl ether (200ml) and vacuum filtration produced a sample of poly(2-alkyl-2-oxazoline) which was characterised using NMR and SEC. **PMeOx** GPC (DMAc): M_n = 9.8 kg/mol (PDI 1.52); ^{1}H NMR ($CDCl_3$, 298 K): 3.47 (br, 4H, (N-C*H*$_2$C*H*$_2$-O)); 2.12 (br, 3H, (C*H*$_3$)). **PEtOx** GPC (DMAc): M_n = 8.2 kg/mol (PDI 1.69); ^{1}H NMR ($CDCl_3$, 298 K): 3.46 (br, 4H, (N-C*H*$_2$C*H*$_2$)); 2.36 (br, 2H, (C*H*$_2$CH$_3$)); 1.24 (br, 3H, (CH$_2$C*H*$_3$)). **P*i*PrOx** GPC (DMAc): M_n = 2.8 kg/mol (PDI 1.09); ^{1}H NMR ($CDCl_3$, 298 K): 3.45 (br, 4H, (N-C*H*$_2$C*H*$_2$)); 2.79 (br, 1H, (C*H*(CH$_3$)$_2$)); 1.10 (br, 6H, (CH(C*H*$_3$)$_2$)). **P*n*BuOx** GPC (DMAc): M_n = 7.2 kg/mol (PDI 1.49); ^{1}H NMR ($CDCl_3$, 298 K): 3.44 (br, 4H, (N-C*H*$_2$C*H*$_2$)); 2.28 (br, 2H, (C*H*$_2$CH$_2$CH$_2$CH$_3$)); 1.59 (br, 2H, (CH$_2$C*H*$_2$CH$_2$CH$_3$)); 1.33 (br, 2H, (CH$_2$CH$_2$C*H*$_2$CH$_3$)); 0.92 (br, 3H, (CH$_2$CH$_2$CH$_2$C*H*$_3$)).

2.6. Polymer surface attachment

Each crude polymer solution was split into two portions (2 x 1.5 ml) and to each portion was added triethylamine (0.030 ml) and acetonitrile (1.5 ml). An amine-coated coverslip was then added and the reaction mixture was left overnight at 60°C. The glass slide was then removed, rinsed with copious amounts of ethanol, and dried under a stream of nitrogen.

2.7. Cell culture

16HBE14o- (referred to as 16HBE, a gift from Professor D.C. Gruenert, San Fransisco, USA [25] were cultured in modified Eagle's medium (MEM) plus Glutamax supplemented with 10% heat-inactivated foetal bovine serum (FBS) and penicillin (50 IU/ml)/streptomycin (20µg/ml). MRC5 fibroblasts were cultured in Dulbecco's MEM (DMEM) supplemented with 10% FBS and penicillin (50 IU/ml)/streptomycin (20µg/ml), L-glutamine (2mM), sodium pyruvate (1mM), and non-essential amino acids (1mM). In some experiments, 16HBE cells that had been stably transfected with a GFP reporter were used. These were generated by transfection with TransIT-2020® transfection reagent (7.5 µl; Mirus Bio, Madison, USA) containing GFP plasmid (pEGFP-N1; 2.5µg, Clontech, California, USA) in Opti-MEM medium (250ul). After 48h, selection medium containing G418 (600 µg/ml) was added and maintained until cells stably expressing GFP were obtained. Once the majority of cells were GFP-labelled, the G418 concentration was reduced to 200µg/ml and cells were FACs sorted using FACSAria (BD biosciences, New Jersey, USA).

2.8. Cell adhesion assay

To create wells on the functionalised glass coverslips, autoclaved cloning rings (6.4mm diameter, Sigma-Aldrich) were attached to polymer-coated glass coverslips using silicon grease (Sigma-Aldrich) and then placed into a 6 well plate or attached to a glass slide ready for cell culture. A single cell suspension of 16HBE or MRC5 cells was prepared by trypsinisation and incubated with Calcein AM for 0.5h in the dark at room temperature. The cells were then washed 3X by centrifugation to remove excess dye. The Calcein AM stained cells ($1x10^4$) were added to the cloning rings and incubated for 1h before the cloning rings were removed and the petri dish was flooded with media. The dish was then gently agitated

to remove non-adherent cells, the medium removed and the slide imaged at 5X magnification (Leica DMI 6000B, with heated chamber). The number of adherent cells were counted using Image J software.

2.9. Cell motility assay

Initial experiments were carried out to optimise the density of cells needed to allow attachment of single cells. After 1 h, the wells were washed to remove non-adherent cells and cell motility was monitored using time-lapse microscopy with images taken at 10 min intervals over a 17 hour period. The movements of 10 cells per well were monitored and the distance travelled was measured using Image J software with the mtrackj plugin.

2.10. Immunofluorescent staining

Cells were initially seeded at $1x10^5$and grown for 5 days within the cloning rings. The cells were then fixed in 4% paraformaldehyde. 16HBE cells were immunofluorescently stained for the tight junction protein, zona-occludin-1 (ZO-1), using an anti-ZO-1 mouse IgG1antibody conjugated to Alexa 647® (Innova Bioscience, Cambridge, UK). The actin cytoskeleton of MRC5 cells was immunofluorescently stained using Acti-stain™ 555 fluorescent phalloidin (cytoskeleton, Inc, Denver, USA). Cell nuclei were visualised using DAPI contained in Prolong Gold mounting solution before being visualised by confocal microscopy (Leica SP5 laser scanning confocal microscope).

2.11. Cell adhesion and selectivity

MRC5 cells ($5x10^3$) were loaded with Cell Tracker Orange and 16HBE cells ($5x10^3$) stably transfected with GFP were seeded together within a cloning ring. Following 1h incubation, the cloning ring was removed and cells washed gently to remove non-adherent cells. The

medium was replaced and the cells were grown for 2 days before being imaged at 5X magnification; cell numbers were counted using Image J and the cell counter plugin.

2.12. Statistics

Data were analysed using two-way ANOVA with Dunnets multiple comparisons test.

3.0 Results

3.1 Functionalisation of glass slides with 2-alkyl-2-oxazoline polymers

For covalent attachment of the poly(2-alkyl-2-oxazoline) to glass slides, the polymers were synthesised and terminated using amine-functionalised glass coverslips. The polymers were characterised using XPS which confirmed the presence of the poly(2-alkyl-2-oxazoline)s. This was accomplished by comparing the relevant carbon-carbon bond energies to carbon nitrogen bond energies. Each batch of surface modifed slides was also characterised using contact angle goniometry which provided a rapid method to determine that the coating was successful. As expected, there was an increase in surface hydrophobicity as the poly(2-oxazoline) series become more hydrophobic (Fig 1). For each polymer type, there was no significant difference in contact angle over the three batches (Fig 2) indicating that the method produced surfaces with reproducible properties.

3.2 Biocompatilibility of polymers

To evaluate the biocompatibility of the polymer surfaces, the growth of bronchial epithelial (16HBE) and fibroblast (MRC5) cell lines was assessed. In all cases, the cells grew to confluence on the functionalised glass coverslips, although the time taken to achieve confluence on each surface varied for both cell types (see below). Immunofluorescent staining of the confluent cell layers showed that the cell morphology was not affected by the oxazoline surfaces, with the 16HBE cells being arranged in a typical cobble-stone pattern and forming tight junctions, whereas the MRC5 cells were spindle shaped with their actin filaments arranged longitudinally throughout each cell (Fig 3). There was no significant difference in biocompatibility between batches of each oxazoline polymer modified glass surface.

As the time taken to achieve confluence on the different polymers varied, the ability of the polymers to support cell adhesion, motility and proliferation was further characterised. Assessment of cell adhesion showed that, with the exception of MRC5 cells plated on PMeOx, there was a significant increase in cell adhesion using the polymer-modified slides compared to the untreated control glass surface slides for both cell types (Fig 4). However, overall, the 16HBE cells were more adhesive than MRC5 cells on all surfaces. 16HBE cells showed a 5-6 fold increase in adhesion compared to the control of untreated glass slides, with the PMeOx surface showing the largest fold increase of 6.2 ± 3.3. The MRC5 cells also demonstrated the greatest fold increase in adhesion on the PEtOx polymer (5.3 ± 1.6 vs control). The greatest difference in adhesion between the two cell types was seen on PMeOx with 16HBEs having a 6.2 ± 3.3 fold increase whereas the MRC5 cells only had a 2.5 ± 1.7 fold increase.

The motility of the cells was also investigated. In contrast with the adhesion assays where the polymers supported increased adhesion, in the cell motility assays their overall effect was suppressive (Fig 5). Furthermore, while epithelial cells were more adhesive, in the motility assays the MRC5 fibroblasts were the more motile cell type. For 16HBE cells, the most hydrophilic polymer surface, PMeOx, caused a significant reduction in cell motility compared with the plain glass control or either the more hydrophobic P*i*PrOx or P*n*BuOx surfaces. While the polymers also tended to reduce the motility of MRC5 cells, it was the more hydrophobic PEtOx, P*i*PrOx and P*n*BuOx surfaces that supported significantly less motility than the plain glass control or PMeOx which provided weaker adhesive surfaces for the MRC5 cells.

To determine whether the differences in cell adhesion and motility could be used to select for a specific cell type from a mixture of cells, equal numbers of 16HBEs and MRC5 cells were allowed to adhere for 1h before the non-adherent cells were removed by washing and the remaining cells left to grow for 2 days. Cell counting showed that all the polymer surfaces had significantly greater total cell numbers than the glass control (Figure 6A). The largest total cell count was found on the P*i*PrOx surface, which had significantly more cells than the plain glass control or the most hydrophobic P*n*BuOx surface. Through use of fluorescently labelled cells, it was also possible to discriminate cell types on each polymer surface. The P*i*PrOx surface had a significantly greater number of both epithelial and fibroblast cells compared to the control (Fig 6C). This surface also had a significantly greater number of 16HBE cells than either PMeOx or P*n*BuOx. The P*n*BuOx was the least preferred polymer surface for 16HBE cells whereas its effect on fibroblast numbers was less notable, thus favouring selection of fibroblasts on this surface.

4.0 Discussion

In this study, we synthesised a series of oxazoline polymers and demonstrated their biocompatibility as cell culture surfaces for 16HBE and MRC5 lung cell lines. The synthetic procedure for the polymers was shown to be reproducible and scaleable. For covalent attachment of the poly(2-alkyl-2-oxazoline) to glass slides, we adapted a procedure from work reported previously [27]. Thus, rather than terminating the polymers using a silane linker and then attaching them to a glass slides, the polymers were terminated using amine functionalised glass coverslips. Although the final structure of the brush surface was the same as that described previously, the alternative ordering of the method facilitated a reproducible and scaleable procedure allowing synthesis of several batches of coated slides in reasonable quantity. This protocol also negated the need to purify the polymers before attachment, which

for P*i*PrOx and P*n*BuOx is difficult since they do not precipitate easily and dialysis is unsuitable as the silane linker is water sensitive. To demonstrate reproducibility we synthesised three batches of polymer coated glass slides for use in the cell testing. Each batch was characterised using contact angles which provided a rapid method to determine that the coating was successful. By monitoring the contact angles, we confirmed the reproducibility of the synthetic method and this was reinforced by the biocompatibility data where no significant differences in cell behaviour were observed between batches.

Both 16HBE and MRC5 cell lines were able to grow to confluence on the series of polymer surfaces. This extends the work of Chang *et al* who have shown that primary sheep and rat fibrocytes and HUVECs will grow on PEtOx covalently attached to glass [24]. In the present study, immunofluorescent staining of the confluent cell layers showed 16HBE cells had formed tight junctions and that the fibroblasts had well organised actin filaments, suggesting that the structural organization of the cells was not affected by growth on the polymers. However, it was noted that the time taken to achieve confluence differed for each polymer, suggesting that the degree of hydrophobicity of the polymer surface affected cell functions. Zhang *et al* have shown that HUVECs will adhere and spread on PMeOx and P*n*PrOx oxazoline bottle-brush brushes covalently attached to glass coverslips, although in this case the surfaces were pre-coated with fibronectin [19]. In the present study, the oxazoline polymers were not pre-coated with fibronectin, although it is possible that some of the properties of the polymer surfaces reflected their ability to bind fibronectin or other proteins from the serum-containing medium used in our studies. However, this difference alone is unlikely to explain the differences in the ability of the polymers to support epithelial cells or fibroblasts in the adhesion, motility and growth assays. Our studies demonstrating that polymer-coated glass substrates support cell adhesion conflict with other studies which have

shown that PMeOx-coated surfaces inhibited bacterial binding, so-called anti-fouling behaviour [28]. However, in view of the marked differences in the structural properties of bacterial cell walls and mammalian cell membranes, it is not unreasonable to expect differences in binding. Another conflicting study reported that fibroblast adhesion was reduced when commercially available, high molar mass PEtOx was immobilized onto glass plates by photochemical grafting [29]. However, in this case, adhesion was measured using single cell force microscopy over a period of up to just 180 seconds. In contrast, our adhesion studies were performed over a longer time period (1 hour) suggesting that initial binding may not reflect more stable binding which can be facilitated by formation of adhesive complexes such as integrins. However, we cannot exclude the possibility that methodological differences could also explain the differing observations. In the current study surfaces contained only a low density of polymer as indicated by the lack of any polymer signals when we attempted to characterise the surfaces by reflective FT-IR. In contrast, the previous study was based on use of ill-defined high molar mass PEtOx (Aquazol).

Adhesion to a tissue culture surface is required for the viability, motility and proliferation of epithelial cells and fibroblasts. Initial interaction of single cells with the surface allows spreading and formation of integrin-meditated contacts which facilitate cell motility. In the case of epithelial cells, when neighbouring cells come together, cell-cell contacts are formed and growth of the epithelial cells occurs in islands (see eg. Fig 6C). In contrast, as reflected in our adhesion and motility data, fibroblasts are a less adhesive and more motile cell type. These properties allow them to play an important role in wound healing. It was of interest that adhesion of 16HBE cells to the polymers tended to decrease as the hydrophobicity of the polymers increased, whereas the effect of polymer hydrophobicity on motility was reversed with PMeOx supporting least epithelial cell motility (ie. more adhesion = less motility). In

contrast, the PMeOx supported most fibroblast motility and was correspondingly less adhesive for these cells.

The observed differences in cell adhesion and motility suggested that the properties of the polymer surfaces could be used to preferentially select for a specific cell type. However, the best surface for supporting epithelial and fibroblast cell growth was P*i*PrOx which supports both cell adhesion and motility, perhaps reflecting the importance of both processes for cell growth. Zhang *et al* have shown that protein absorption is lower on PMeOx and PEtOx at 6ng/cm^2 compared to P*n*PrOx at 90ng/cm^2 [19]. This lower protein absorption may also affect cell growth on the PMeOx and PEtOx surfaces compared with P*i*PrOx. Although the lowest total cell number in the co-culture was obtained using the P*n*BuOx polymer surface, this was still greater than seen on plain glass. Furthermore, it was the reduction in 16HBE cells that provided the biggest difference between P*n*BuOx and the other polymers. Therefore, it may be possible to use serial passage the P*n*BuOx polymer to select for a higher proportion of fibroblasts from a mixed culture (eg. cells obtained from dissociation of lung tissue).

PEtOx and P*i*PrOx have been shown to display thermoresponsive behaviour in solution [30] similar to that of PN*i*PAAm, a polymer which also displays this behavour and is commonly used to synthesise thermoresponsive tissue culture surfaces [31]. We attempted to measure T-dependent contact angles on P*i*PrOx surfaces but observed no statistically significant variation with temperature. As suggested above, we suspect that this may be due to the low density of the P*i*PrOx chains leading to only a very small change in surface wettability [32]. Such density dependance has also been seen for PN*i*PAAm coated surfaces [33]. Although we did not observe the thermoresponsive behaviour using the oxazoline polymers attached to

the glass slides, there is the potential to further develop these surfaces for cell sheet growth and harvesting.

5. Conclusions

A scalable and reproducible method for synthesising a series of oxazoline polymers on glass surfaces has been established and their use as cell-compatible growth surfaces has been demonstrated. The polymers exhibit differences in support of epithelial cell and fibroblast adhesion, motility and growth offering potential for development of cell selective surfaces. There is the opportunity to further functionalise the polymers giving a large scope for more tailored cell culture surfaces.

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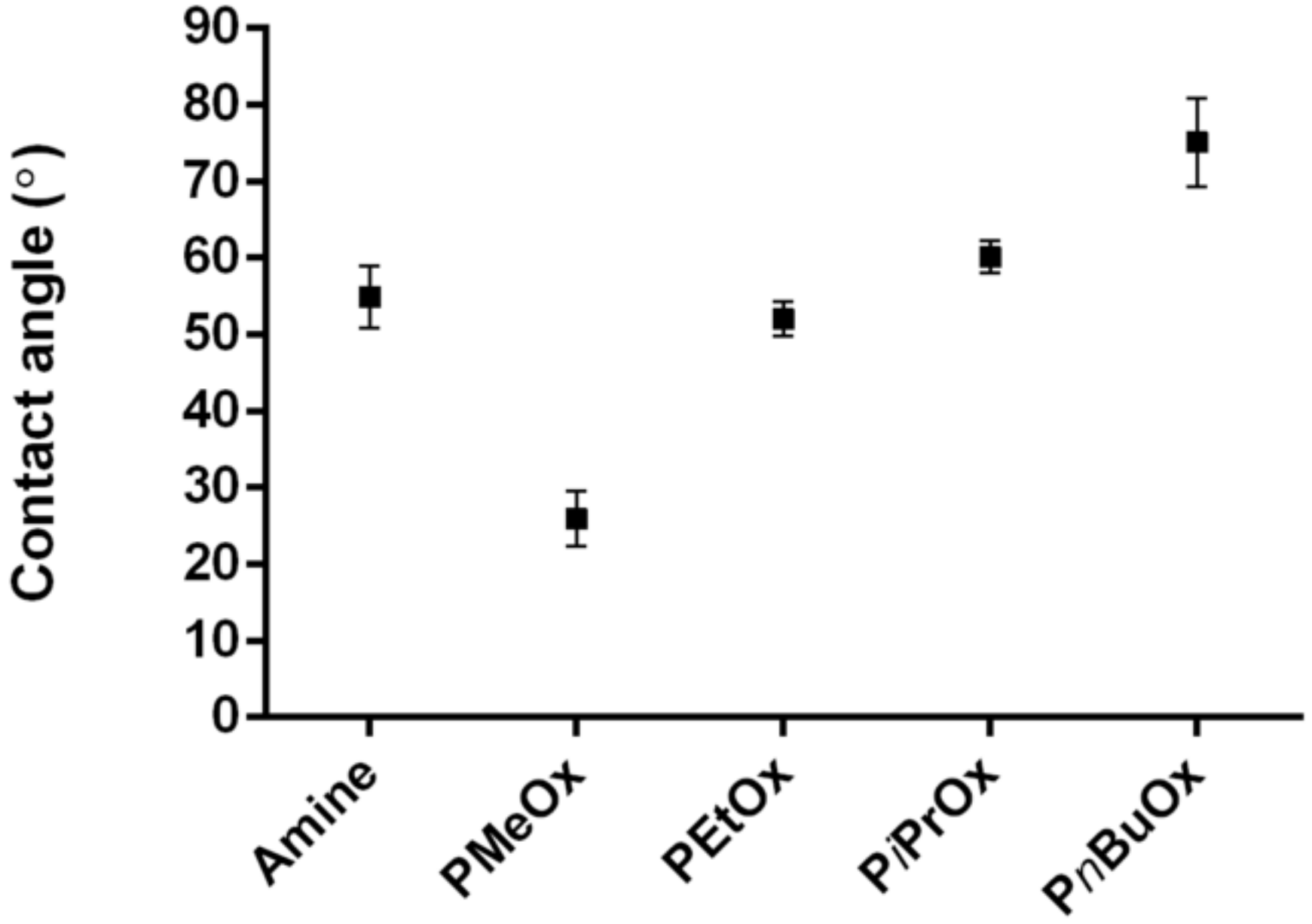
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50
40
30
20
10
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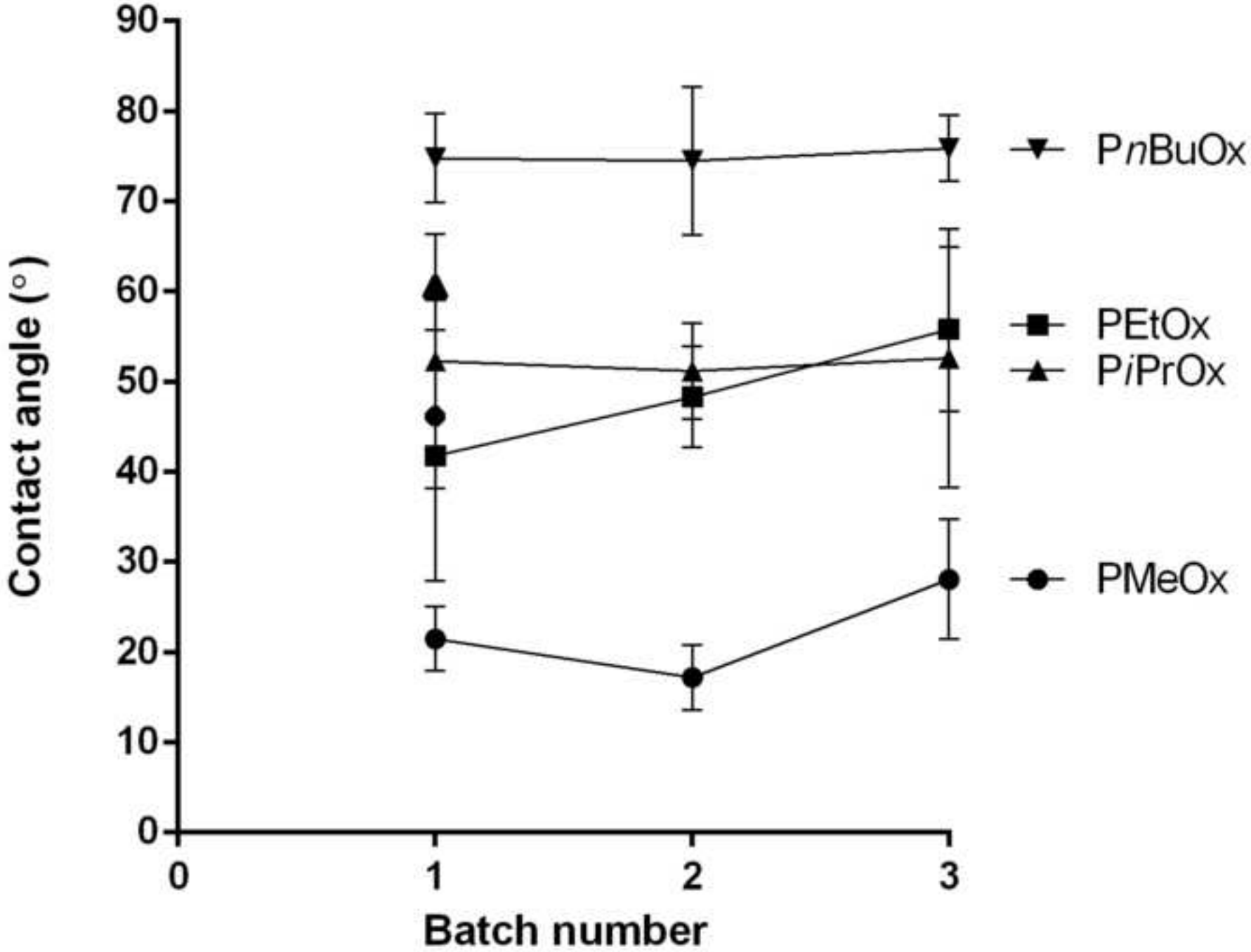
PEtOx
PiPrOx
PnBuOx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2
3
Batch number
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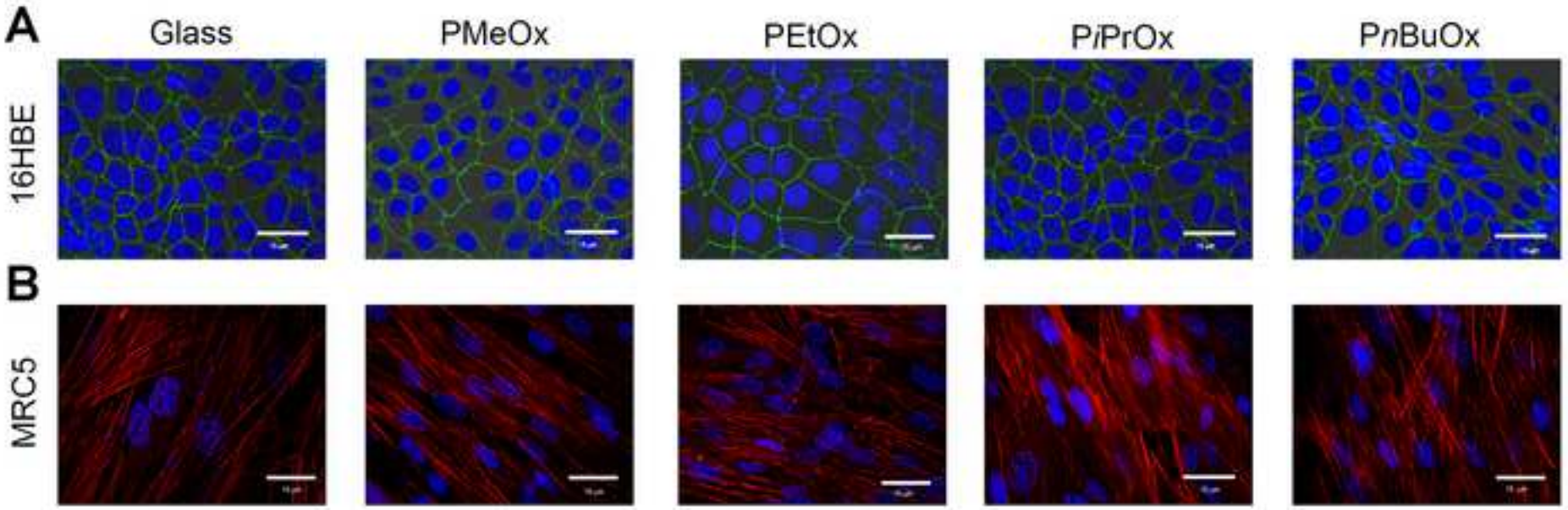
PMeOx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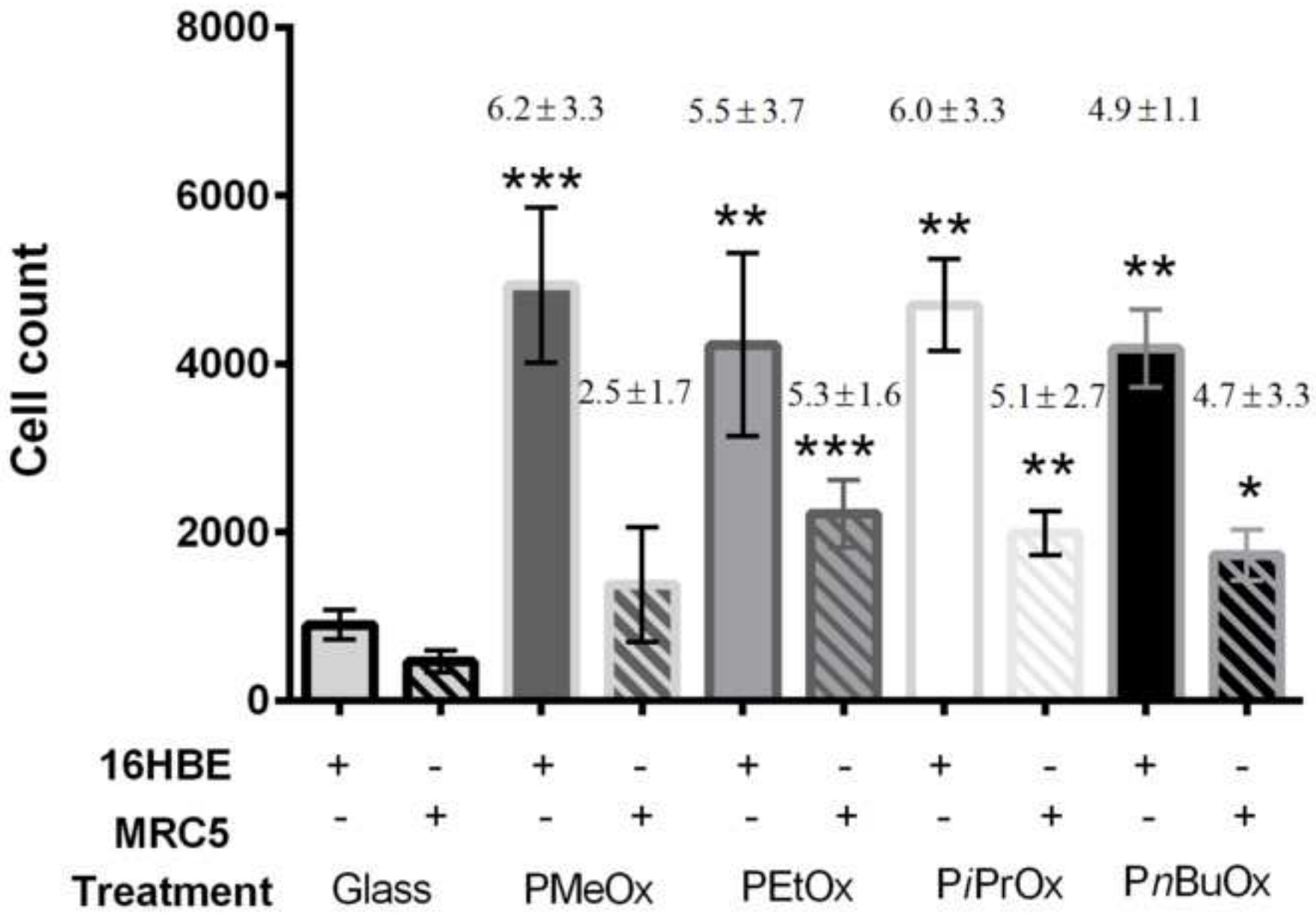
16HBE
B
MRC5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4000
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5.5±3.7
6.0±3.3
4.9±1.1
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2.5±1.7
5.3±1.6
5.1±2.7
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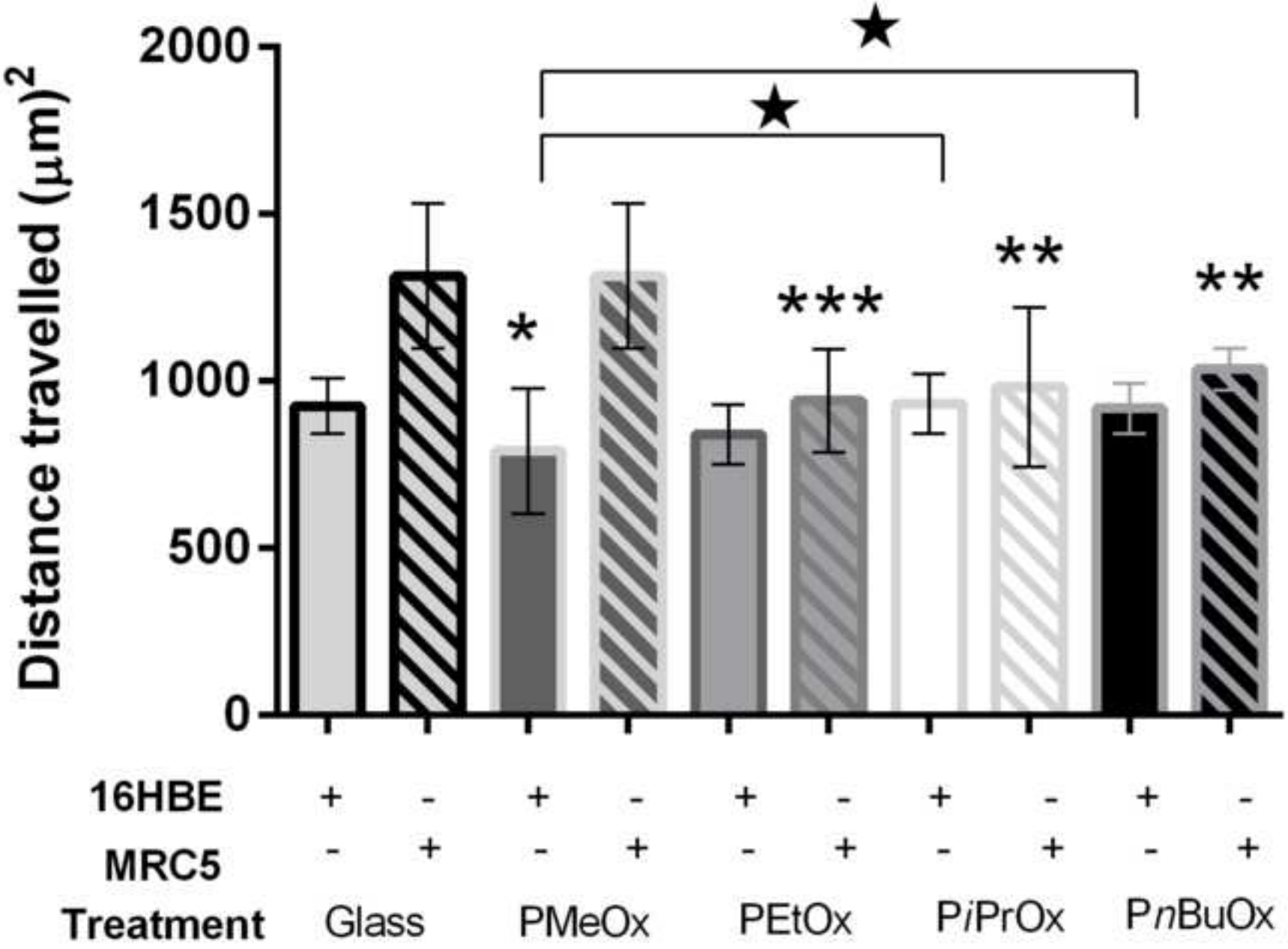
Treatment Glass PMeOx PEtOx PiPrOx PnBuOx


Distance travelled (µm)²
2000
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0
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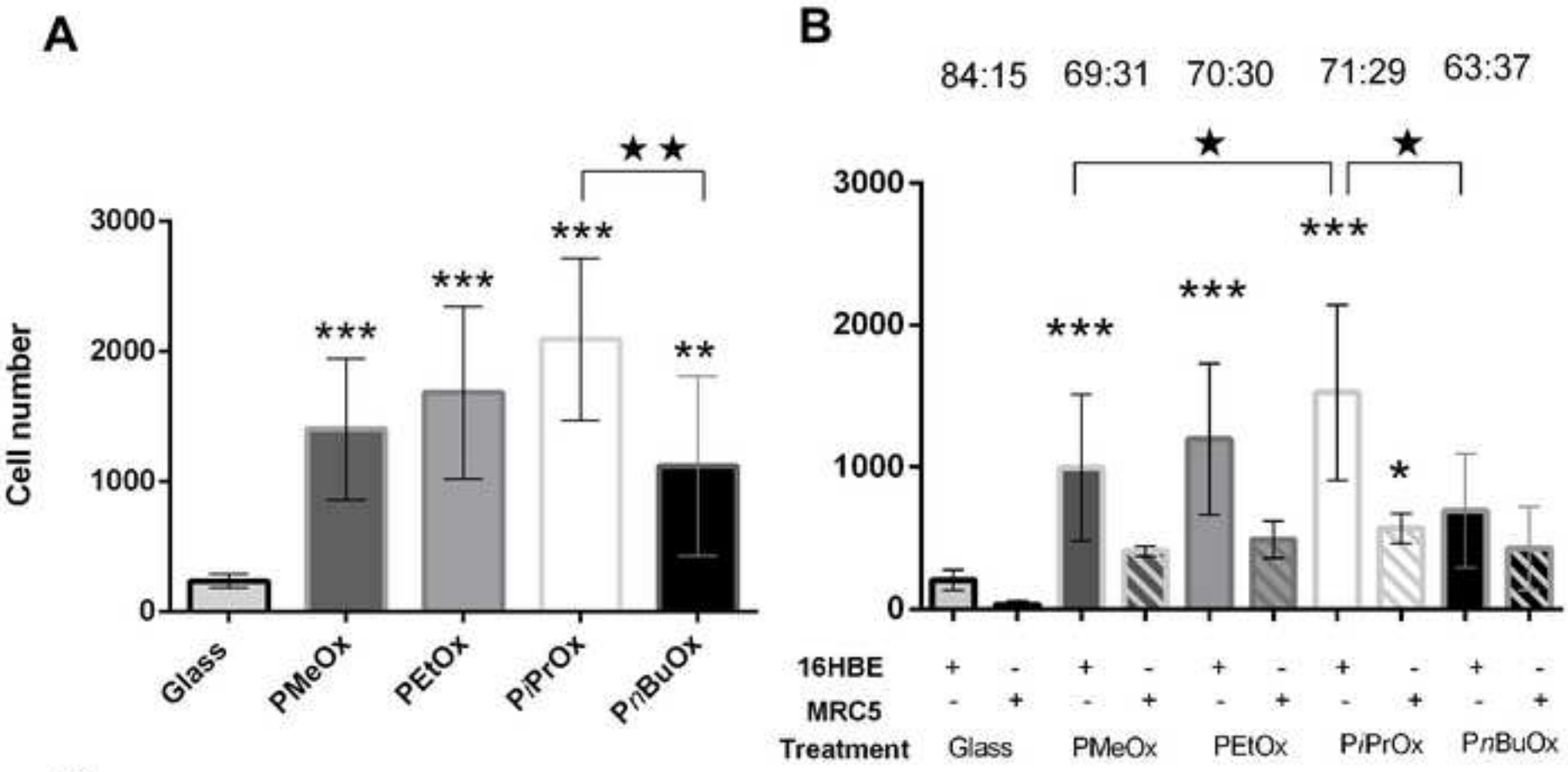
16HBE + - + - + - + - + -
MRC5 - + - + - + - + - +
Treatment Glass PMeOx PEtOx PiPrOx PnBuOx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Cell number
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★ ★
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PEtOx
PiPrOx
PnBuOx
B
84:15
69:31
70:30
71:29
63:37
★
★
3000
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Treatment
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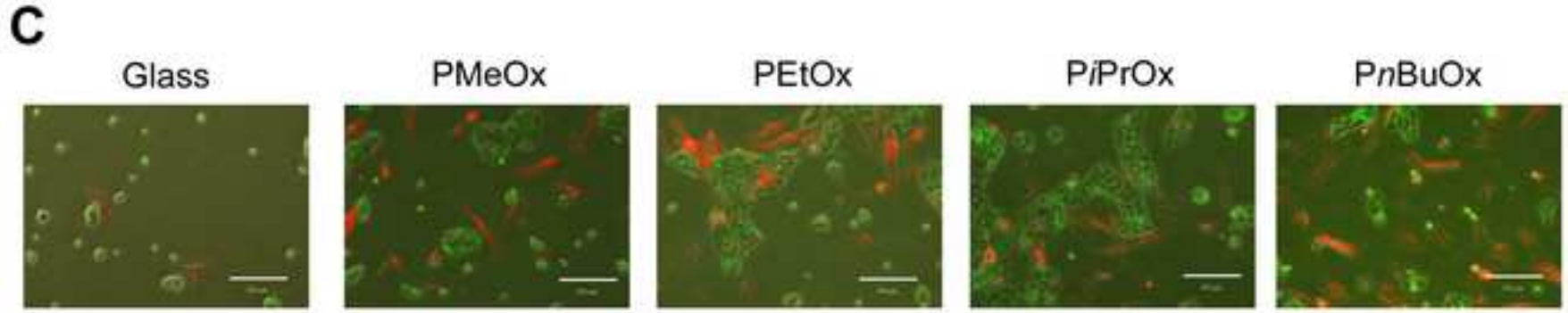
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Glass
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Figure Captions

Fig 1. Hydrophobicity of oxazoline surfaces. Contact angle goniometry was performed on the initial amine modified glass coverslip (amine) and those to which the oxazoline polymers (PMeOx, PEtOx, P*i*PrOx and P*n*BuOX) had been covalently attached. Data are mean ± SD, n=10.

Fig 2. Batch reproducibility of the oxazoline surfaces. Contact angles were measured for 3 individual batches of the polymer modified glass slides as described in Figure 1. Data are mean ± SD, *n* = 3. Statistical significance was analysed by two-way ANOVA and Tukey's multiple comparisons test and showed no significant difference between batches.

Fig 3. Biocompatibility of oxazoline surfaces. Cells were grown to confluence on control glass slides or slides modified with the indicated oxazoline polymer surfaces and then fixed for immunofluorescent staining. 16HBE cells were immunostained for the tight junction protein, ZO-1, using DAPI as counter stain (A). The actin cytoskeleton of MRC5 fibroblasts was stained using acti-stain^{TM} 555fluorescent phalloidin with DAPI as counterstain (B). Scale bar represents 15 µm. Results shown are representative images of each condition performed in 3 independent experiments.

Fig 4. Adhesion of 16HBE and MRC5 cells to the oxazoline polymer surfaces. Cells were fluorescently labelled with Calcein AM and then added to slides modified with the indicated oxazoline polymer surfaces or uncoated glass slides (as a control) for 1 h. After washing, adherent cells were imaged using fluorescence microscopy and counted with Image J. Data are mean ± SD, *n* = 3. Statistical analysis was performed using two-way ANOVA with Dunnett's correction for multiple comparisons. Numbers above bar charts indicate the fold

increase compared to the untreated glass slide control. *p<0.05, **p<0.01 ***p<0.001 versus control.

Fig 5. Motility of 16HBE and MRC5 cells on the oxazoline polymer surfaces. Cells were added to control glass slides or slides modified with the indicated oxazoline polymer surfaces for 1h. After washing, cell motility was monitored using time-lapse microscopy. The distance travelled by 10 cells per experiment was measured using Image J software. Bars represent mean ± SD, n = 3. Statistical analysis was performed using two-way ANOVA and Dunnett's test for multiple comparison. *p<0.05, **p<0.01, ***p<0.001 versus control and $^{\star}p$<0.05 between the polymer surfaces.

Fig 6. Co-culture of 16HBE and MRC5 cells on oxazoline surfaces. Fluorescently labelled MRC5 (orange) and 16HBE (green) cells were seeded in equal numbers and allowed to attach for 1h before washing to remove non-adherent cells. The medium was replaced and the cells grown for 2 days before the number of epithelial cells and fibroblasts were counted. Panel A shows the total cell number on each polymer surface and panel B shows the number of 16HBE and MRC5 cells on each polymer surface. Panel C shows microscope images of the co-culture on the polymer surfaces. Scale bar = 250 μm. Results are mean ± SD. for n = 3 (A, B) or representative images of n = 3 (C) Statistical analysis was performed using two-way ANOVA and Dunnet's test for multiple comparisons. * shows statistical differences compared to the control. *p<0.05, **p<0.01, ***p<0.001 versus control and *p<0.05, **p<0.01 between the polymer surfaces.