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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 (16HBE14o-) 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.

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

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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 400 μm 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 1 μL 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 (2.22 M), ethanolamine (163 ml, 2.66 M) and zinc acetate (24 g, 0.111 M) were stirred for 20 h 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 (300 Mhz, $CDCl_3$, 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 (300 Mhz, $CDCl_3$, 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 (300 Mhz, $CDCl_3$, 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 (300 Mhz, $CDCl_3$, 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 × 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 1 h, 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 (38 μl) in freshly distilled ethanol (5 ml) for 1 h. The slides were removed and washed with copious amounts of ethanol before being heated to 80°C for a further 1 h.

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 (22 ml), 2-ethyl-2-oxazoline (26.22 ml), 2-isopropyl-2-oxazoline (31.07 ml) and 2-butyl-2-oxazoline (35.91 ml). Briefly, acetonitrile (44 ml), 2-alkyl-2-oxazoline (259.81 mmol) and methyl *p*-toluenesulfonate (0.261 ml, 1.73 mmol) were thoroughly mixed and then split into aliquots of 3 ml 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 (1 ml) was removed and sat. KOH in methanol was added (100 μl) and the sample was stirred overnight at 60 °C. Subsequent precipitation in ice cold diethyl ether (200 ml) 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–CH_2CH_2–O)); 2.12 (br, 3H, (CH_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–CH_2CH_2)); 2.36 (br, 2H, (CH_2CH_3)); 1.24 (br, 3H, (CH_2CH_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–CH_2CH_2)); 2.79 (br, 1H, ($CH(CH_3)_2$)); 1.10 (br, 6H, ($CH(CH_3)_2$)). **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–CH_2CH_2)); 2.28 (br, 2H, ($CH_2CH_2CH_2CH_3$)); 1.59 (br, 2H, ($CH_2CH_2CH_2CH_3$)); 1.33 (br, 2H, ($CH_2CH_2CH_2CH_3$)); 0.92 (br, 3H, ($CH_2CH_2CH_2CH_3$)).

2.6. Polymer surface attachment

Each crude polymer solution was split into two portions