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Evaluation of the In Vitro Cytotoxicity and Modulation of the Inflammatory Response by the Bioresorbable Polymers Poly(D,L-lactide-co-glycolide) and Poly(L-lactide-co-glycolide)

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ABSTRACT

Bioresorbable polymers composed of poly(D,L-lactide-co-glycolide) (PDLLGA) and poly(L-lactide-co-glycolide) (PLLGA) have become increasingly popular for the preparation of bone substitute constructs. However, there are reports of a delayed inflammatory reaction occurring months or years after implantation. Due to the long polymer degradation times, \textit{in vitro} tests carried out at physiological temperature, 37°C, tend to assess only the short-term biocompatibility of these materials. The aim of this work is to develop an \textit{in vitro} protocol that can be used to assess the long-term cytotoxicity of bioresorbable polymers in a time efficient manner. This study used a previously developed and validated accelerated degradation protocol to obtain samples of PDLLGA and PLLGA at increasing levels of degradation. Samples were then applied to standard ISO 10993-5 direct contact cytotoxicity testing and it was found that PDLLGA samples showed increasing levels of cytotoxicity at the later stages of degradation, with PLLGA samples demonstrating significantly less cytotoxic behaviour. Following concern that accumulation of acidic degradation products in a closed multi-well culture environment could overestimate cytotoxicity, we developed and validated a new dynamic flow culture methodology, for testing the cytotoxicity of these degradable materials, by adapting a commercial “organ on a chip” flow culture system, Quasi Vivo\textsuperscript{®}. In addition to cytotoxicity testing, we have carried out profiling of inflammatory cytokines released by cells in response to degraded PDLLGA and PLLGA, and have suggested mechanism by which lactide-based bioresorbable materials could modulate the inflammatory response through the G-protein coupled receptor (GPCR), hydroxycarboxylic acid receptor 1 (HCA\textsubscript{1}).

Keywords
Poly(lactide-co-glycolide); Biocompatibility; Cytotoxicity; Quasi Vivo; Lactate
1. Introduction

Bioresorbable polymers composed of poly(lactide) (PLA), poly(glycolide) (PGA) and their related copolymers are commonly used in orthopaedic applications to help regenerate and repair injured bone tissue. Upon implantation, polymers degrade via hydrolytic degradation, gradually transferring the load to the bone tissue, with metabolic pathways used to excrete the degradation products from the body[1–3]. The main benefit of bioresorbable polymers is the ability to tailor their physicochemical and mechanical properties to meet the desired needs of the application. Poly(L-lactide) (PLLA) is commonly used for orthopaedic applications, however, reports of long polymer degradation times, in excess of 5 years, has led to research into faster degrading polymers, allowing replacement by regenerating tissue over more realistic timeframes [4–8]. There is growing interest into the copolymerisation of poly(D,L-lactide) (PDLLA) and PLLA with PGA forming poly(D,L-lactide-co-glycolide) (PDLLGA) and poly(L-lactide-co-glycolide) (PLLGA). The inherent properties of the amorphous PDLLGA and semi-crystalline PLLGA, give the copolymers different characteristics and their mechanical, physiochemical and degradation properties can be controlled by altering their monomer unit ratios.

The in vivo host response to implanted biomaterials is important to be considered during the development of bioresorbable polymers. Upon implantation, a host reaction is triggered and results in a cascade of regulated events. Following implantation, a provisional matrix is formed by protein adsorption on the surface of the biomaterial and is rich in cytokines, chemoattractants and growth factors. Acute inflammation follows and is characterised by the presence of neutrophils, whose role involves the removal of cellular debris via phagocytosis and the expression of cytokines, which are involved in the recruitment of macrophages, such as macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) [9–13]. This phase is followed by chronic inflammation, where inflammatory cells, such as monocytes, are recruited to the site of implantation. The acute and chronic inflammation phases are relatively short, usually lasting no longer than two weeks. The resolution of these phases leads to the formation of granulation tissue, which can be identified by the presence of macrophages, fibroblasts, and the proliferation of small blood vessels. The foreign body reaction (FBR) is identified by the presence of macrophages and foreign body giant cells at the surface of the implant, together with the components of the granulation tissue. The cellular composition of the FBR is largely determined by the form, topography, and surface-to-volume ratio of the implanted biomaterial and can persist for its lifetime. The final stage of the healing process is fibrous capsule formation surrounding the biomaterial. This acts as protective barrier isolating the FBR and the biomaterial from the host tissue [13,14]. This is the body’s natural healing mechanism and is essential for the successful integration of the implanted biomaterial with its host environment. The progression of these stages occurs via complex cellular interactions, with crosstalk between different cascade systems controlling the cellular response and behaviour. Development of a better understanding of these complex interactions will enhance the progression of suitable bioresorbable polymers for use as medical devices.

Biocompatibility is used to describe the desired interaction of a biomaterial with its host environment, whereby the biomaterial or degradation products should not cause any adverse reactions or cytotoxicity after implantation [15]. PLA, PGA, and their related copolymers are widely considered biocompatible, however, throughout the literature there is evidence of a delayed inflammatory reaction occurring months or years after implantation. These reactions generally
present as a non-specific FBR and can range in severity [5,16–20]. The occurrence of this reaction is not well understood as it is not specific to polymer type, implant location or subject [5]. Numerous reasons for the reported incompatibility have been hypothesised, which include the presence of crystalline particle debris, the accumulation of degradation products, the acidic environment created by the release of acidic by-products, the geometry of the implant and the implant location [21–24]. However, it is difficult to determine the aetiology of this response as it is difficult to isolate the factors that contribute to this response.

Medical devices must undergo a series of tests to ensure they are safe for clinical use, with these tests regulated by the International Organisation for Standardisation (ISO) [25,26]. Cytotoxicity analysis is required for every medical device and is generally the first step of in vitro biocompatibility testing. These tests provide a highly effective method to determine the cytotoxicity of a material or substance on living cells, however, their use is limited as they cannot be used to determine the cause of cellular death. Testing of the in vitro immune response at the early stages of biomaterial development is currently not incorporated within standard procedures. Gaining insight into the in vitro cell response to biomaterials would allow for early detection and prediction of potential adverse reactions. Protein-based arrays can be used as a useful method to evaluate how biomaterials interact with different cells within the FBR [27]. The profiling of cytokines expressed by cells in contact with biomaterials can be determined using antibody arrays and an enzyme-linked immunosorbent assay (ELISA).

In vitro studies play an important role in the prediction of in vivo behaviour by allowing rapid screening of biomaterials prior to animal trials. However, in vitro tests tend to assess the short-term effects, with the long-term biocompatibility assessed using in vivo trials. The development of extended in vitro biocompatibility testing for degradable biomaterials would be beneficial, as it would allow the early identification of potential issues before progressing to animal or clinical studies and would also advance the understanding of why adverse reactions occur. Despite clinical studies demonstrating adverse reactions to bioresorbable polymers, there are few in vitro studies that corroborate these results. The aim of this work is to develop an in vitro protocol that can be used to assess the cytotoxicity of bioresorbable polymers in a time efficient manner.

Current in vitro ISO standard tests for cytotoxicity are carried out at 37°C in cell culture media (or other suitable diluent), in closed, static multi-well culture vessels. These tests only assess the short-term biocompatibility, whereas late-stage degradation, can take months to occur at physiological temperature. This timescale is incompatible with cell culture-based assays.

In this study, we have used a previously developed and validated accelerated degradation protocol to obtain polymer samples at increasing levels of degradation [28]. This utilised an elevated temperature of 47°C, i.e. 10°C above physiological temperature. This previous study also compared elevated temperatures of 57°C and 70°C and concluded 47°C was the optimum temperature owing to the fact that it was below the polymer’s Tg, and produced consistent results over practicable timescales. Briefly, polymers were incubated in sterile phosphate buffered saline at 47°C, and samples were removed at predetermined time points, informed by physicochemical characterisation. The samples were then applied to standard ISO 10993-5 direct contact cytotoxicity testing [29]. Additionally, we have developed a dynamic flow culture methodology for testing these degradable materials, following concern that accumulation of acidic degradation products in closed multi-well culture could overestimate cytotoxicity. In an in vivo physiological environment, diffusion of degradation products will occur, and there will be an exchange of tissue fluid in the peri-implant microenvironment. To replicate the in vivo environment and increase physiological relevance, we
have adapted a commercial “organ on a chip” flow culture system, Quasi Vivo®, for testing the polymer samples.

In addition to cytotoxicity testing, we have carried out profiling of inflammatory cytokines released by cells in response to degraded PDLLGA and PLLGA, and have proposed a suggested mechanism by which lactide-based bioresorbable materials could modulate the inflammatory response through the G-protein coupled receptor (GPCR), Hydroxycarboxylic acid receptor 1 (HCA1).

2. Materials and Methods

2.1 Materials

PDLLGA 85:15 (PURASORB PDLG 8531, Corbion Biomaterials, the Netherlands) and PLLGA 85:15 (PURASORB PLG 8531, Corbion Biomaterials, the Netherlands) in pellet form were used for this study. Materials were stored in a freezer (-20°C) in sealed packaging until use.

2.2 Sample Preparation

PDLLGA and PLLGA pellets were compression moulded (Collins P200P), at 180°C for 6.5 minutes, into sheets with a volume of 100 x 100 x 1 mm³. PLLGA compression moulded sheets were then annealed in an oven for 4 hours at 100°C (PDLLGA was not annealed, as this was considered unnecessary due to its amorphous nature). A laser cutting machine (FB 1800 50W) was used to cut the PDLLGA and PLLGA sheets into disc-shaped samples with an 8mm diameter. Samples were sterilised using electron beam radiation (Steris, Ireland), with a dosage of 20 kGy. For experimentation under static cell culture conditions positive control material was prepared by swelling poly(2-hydroxyethyl methacrylate) (pHEMA) in a solution of 0.1% benzalkonium chloride (BAK) in phosphate buffered saline (PBS), and sterilising by autoclave. BAK is a known cytotoxic substance and hydrogel polymers soaked in BAK have been validated as a suitable positive control material for cytotoxicity testing [30]. The negative controls were non-degraded PDLLGA samples and non-degraded PLLGA samples.

2.3 In Vitro Degradation

PDLLGA and PLLGA samples were separated into three groups, the first group was used for characterisation, the second for tests on L929 murine fibroblasts and the third for tests on RAW264.7 murine macrophages. Under sterile conditions, samples were placed into transwell inserts (Millipore, PIHP01250), immersed in 10 mL of PBS buffer (pH 7.4) and degraded in an oven at 47°C. PDLLGA sample were degraded for 5, 7, 10 and 12 days and PLLGA was degraded for 28, 42, 56 and 70 days.

2.4 Characterisation

Polymers were characterised in a previous study by the authors [28]. PDLLGA and PLLGA were assessed for changes in the pH of the degradation media (PBS buffer), percentage mass loss, percentage swelling and molecular weight loss during degradation at 47°C. Briefly, the pH of the degradation media (PBS buffer) was recorded at each time point. Prior to degradation, the initial mass of the samples was recorded. At each time point the samples (n=5) were retrieved from the PBS buffer and rinsed with distilled water. Surface moisture was removed, and the weight was...
recorded (wet mass). The dry mass was then determined after samples were dried in a vacuum oven for 72 hours at 30°C and 600 mmHg. Equation 1 and Equation 2 were used to determine the percentage mass loss and swelling of the samples, respectively.

\[
\text{% Mass Loss} = \frac{m_0 - m_d}{m_0} \times 100 \quad (\text{Equation 1})
\]

\[
\text{% Swelling} = \frac{m_w - m_d}{m_d} \times 100 \quad (\text{Equation 2})
\]

Where, \(m_0\) = initial mass; \(m_d\) = dry mass and \(m_w\) = wet mass

The number average molecular weight (\(M_n\)) of the samples was determined by gel permeation chromatography (GPC).

2.5 Cell Culture

L-929 murine fibroblasts (ATCC CCL-1) were maintained in Eagle’s minimum essential medium (MEM) containing 10% foetal bovine serum (FBS) and 50 U/mL penicillin/streptomycin (pen strep). RAW264.7 (ATCC TIB-71) murine macrophages were maintained in Dulbecco’s minimum essential medium (DMEM) containing 10% FBS and 50 U/mL pen strep. Both cell lines were cultured under standard conditions of 37°C and 5% CO₂.

2.6 Cell Viability Assay

2.6.1 Static Conditions

Cells were cultured in a 24-well plate for 24 hours in 1 mL of media to allow for a near confluent monolayer. The degraded samples, in the transwell inserts, were placed into the wells of the tissue culture plate, and cells incubated for a further 24 hours at 37°C. After this time, the transwell inserts were removed from the 24-well plate. The supernatants were removed from the cells, placed into microcentrifuge tubes, and stored at -80°C for later analysis (i.e. antibody arrays and ELISA analysis described below). 500 μL of fresh MEM or DMEM were then added to the cells. All experimental steps were carried out under aseptic conditions in a Class II microbiological safety cabinet. All cell culture experiments were carried out in triplicate.

2.6.2 Development of Flow Conditions for Cell Culture using Quasi Vivo® QV500 System

The Quasi Vivo® QV500 system (Kirkstall) is a flow system that can be used to culture cells in a way that better reflects the dynamic nature of the physiological environment. This approach utilises interconnected chambers with cell culture media pumped through the system, allowing cells to be cultured under flow conditions. The components of the QV500 system were connected under sterile conditions in a Class II microbiological safety cabinet. The system is designed to recirculate media; however, this was adapted for the current study to allow for a single pass of media though the system. Nine Quasi Vivo® chambers were connected in series and each sample examined in triplicate (Figure 1). A peristaltic pump (SP-minipump compact, Shenzhen Baoding) with variable speed control was used with the QV500 system. The pump was calibrated in order to achieve a flowrate of
75 μL/min; this flowrate was selected as it was used by Nithiananthan et al [31] when using the QV500 system with fibroblast cell lines.

2.6.2.1 Flow Conditions using Quasi Vivo® QV500 System

Prior to use, glass coverslips (13 mm diameter) were sterilised in ethanol, rinsed in PBS, primed in MEM, and placed into a 24-well plate. L929 fibroblasts were cultured, in static conditions, on coverslips in 1 mL of MEM at 37°C with 5% CO₂, for 24 hours. As per manufacturer’s instructions, before starting the experiment, the QV500 system was washed with PBS buffer for 20 minutes, then primed with cell culture media (MEM) for 20 minutes, with the peristaltic pump set to its maximum flowrate. The media was then removed from the system. Figure 2 shows step-by-step images of the next stages of the experiment, wherein material samples are loaded into each QV500 chamber. The closed system is then transferred to the incubator and operated with a flowrate of 75 μL/min for 24 hours. After this time, coverslips were removed from the system and placed into a 24-well plate, with 500 μL of fresh MEM added, in preparation for the MTT cytotoxicity assay.

![Figure 1. Schematic representation of QV500 system (A) and real image of QV500 system (B)](image)

**Figure 1. Schematic representation of QV500 system (A) and real image of QV500 system (B)**

2.6.3 MTT Cytotoxicity Assay

The viability of the cells is determined by the ability of the mitochondrial enzyme succinate dehydrogenase to metabolically reduce the yellow water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) into blue/ violet formazan crystals. The intensity of the blue/
violet colour is directly related to the number of viable cells [29,32]. Following the treatments described in Section 2.6.1 and Section 2.6.2, 100 µL of a filter-sterilised MTT (5 mg/mL) solution was added to the cells in the 24-well plates. The plates were wrapped in tin foil to protect from light and placed in the incubator at 37°C for 2 hours. Media was then aspirated, and the formazan crystals formed were dissolved in 500 µL of dimethyl sulfoxide (DMSO). The optical density was measured at a wavelength of 570 nm using a FLUOstar Omega microplate reader (BMG Labtech). Equation 3 was used to monitor the changes in cell viability. As stated by ISO 10993-5, if the cell viability is less than 70% the material has cytotoxic potential. The lower the percentage viability the greater the cytotoxic potential of the material. To calculate the percentage viability all the sample values are relative to a “cells only” blank control, as per Equation 3.

\[
Cell \ Viability \ (%) = \frac{OD_s}{OD_b} \times 100 \%
\]  
(Equation 3)

Where: OD_s is the optical density of the test sample extracts and OD_b is the optical density of the “cells only” blanks.

2.7 Antibody Array

The relative expression levels of 40 mouse cytokines from selected samples were determined using a Proteome Profiler™ Mouse Cytokine Array, Panel A (R&D Systems). Four samples of cell conditioned media were selected based on the results of the MTT cytotoxicity assay, in Section 3.2. These included the supernatants collected from L929 fibroblasts and RAW264.7 macrophages after contact with PDLLGA degraded for 10 days and PLLGA degraded for 42 days. These time points were selected at which the materials began to show slight reduction in cell viability. The controls used for this study were the supernatants collected from the L929 fibroblasts and RAW264.7 macrophages cultured in cell medium only. Supernatants from triplicate samples were pooled and prepared according to the manufacturer’s array procedure. The cytokine array was conducted according to the manufacturer’s instructions and chemiluminescence was detected using the Molecular Imager® ChemiDoc™ XRS+ imaging system (BioRad). Images were analysed using Image Studio Lite version 5.2 software.

2.8 ELISA

MCP-1 and MIP-1α, were selected for continued analysis using the DuoSet® ELISA Development System (R&D Systems), based on the results of the antibody array, in Section 3.5. Cell conditioned media, collected under static conditions (Section 2.6.1), were analysed. These samples included the supernatants collected from L929 fibroblasts and RAW264.7 macrophages in contact with PDLLGA non-degraded and degraded for 7, 10 and 12 days at 47°C and PLLGA non-degraded and degraded for 28, 42, 56 and 70 days at 47°C. The controls used were the supernatants collected from the L929 fibroblasts and RAW264.7 macrophages cultured in cell culture medium only. The samples collected from the L929 fibroblasts were analysed for MCP-1 and the samples collected from the RAW264.7 macrophages were analysed for MIP-1α. The ELISA was carried out according to the manufacturer’s instructions. All ELISA analysis was performed in duplicate wells, on three experimental replicates. The optical density was determined using a FLUOstar Omega microplate reader (BMG Labtech), measured at wavelengths of 450 nm and 570 nm. Wavelength correction was calculated by
subtraction of the reading at 570 nm from the reading at 450 nm. The calibration curve was generated using four-parameter logistic (4-PL) curve fit.

2.9 GPCR (HCAR₁) activation and calcium mobilisation assays

GPCR activation was monitored via calcium signalling using the proprietary Fluo-4 Direct TM Calcium Assay Kit. This kit contains a Ca²⁺ indicator, which is loaded into the cells and produces a large fluorescence increase upon binding Ca²⁺. To prevent extrusion of the indicator out of the cell by organic anion transporters, probenecid is included in the kit formulation, and this helps to reduce the baseline signal. Media removal and washing steps are not required with the Fluo-4 Direct TM Calcium Assay Kit, as background fluorescence attributable to the growth media is eliminated by the addition of a suppression dye.

Cells were seeded at a density of 5 x 10⁴ cells/well in black sterile 96-well plates with optical bottom, in 100 µl of the appropriate culture media, and allowed to recover for 24 hours. The Fluo-4 Direct TM indicator was reconstituted as per kit instructions under aseptic conditions and added to each well in an equal volume to culture media. The plate was wrapped in aluminium foil to protect from light, then incubated for 30 minutes at 37°C, and a further 30 minutes at room temperature.

To confirm GPCR receptor activation in response to lactate (and the presence of HCA₁) 15 µM sodium L-lactate was added to indicator loaded cells, and the fluorescence was monitored using a BMG Fluostar Optima® spectrofluorimeter, at wavelengths of 494 nm excitation and 516 nm emission. The response to 10 µL samples of the polymer degradation media (as described in Section 2.3) was also evaluated, with degradation time points of 12 days for PDLGA, and 70 days for PLLGA selected for examination. This analysis was carried out in triplicate. Sterile, calcium free PBS was used as a vehicle control and was not found to have any effect on receptor activation (Supplementary Information, Figure S1).

2.10 Statistics

Statistical analysis was carried out using GraphPad Prism 5.0 software. The results are expressed as the mean ± standard deviation. Statistical analysis for the static conditions MTT assay and ELISA was performed using a one-way ANOVA with a post-hoc Tukey analysis. Statistical significance was set at p < 0.05. Statistical analysis for the Quasi Vivo flow conditions was performed using a t-test. Statistical significance was set at p < 0.05.
3. Results

3.1 Visual Inspection

PDLLGA underwent significant changes to its appearance during degradation and changes occurred without considerable mass loss, where, after 12 days, only 6% mass was lost from the samples (Figure 3a). After 5 days at 47°C the samples became visibly damaged, after 10 days the samples appeared visibly swollen with a hard exterior and viscous liquid interior, and after 12 days the samples were a viscous liquid. In contrast, the appearance of PLLGA (Figure 3b) remained the same until 56 days at 47°C, where the samples changed from a translucent off-white colour to opaque and bright white. Throughout degradation, the samples appeared volumetrically unchanged, however, when mass loss occurred, after 56 days, the samples became fragile and disintegrated into a powder on handling.

![Figure 3. Samples appearance and mass loss of a) PDLLGA and b) PLLGA after degradation at 47°C and drying.](image)

3.2 MTT Cytotoxicity Assay for Static Conditions

The direct cytotoxicity assay (conducted as per ISO 10993-5) on pre-degraded PDLLGA (Figure 4a) showed similar trends in both fibroblasts and macrophages. High cell viability values, between 86 – 106%, were obtained in both cell lines for the 5- and 7- days samples, which showed no significant difference to the negative control. However, a significant decrease compared to the negative control, was observed after 10 days, as viability was reduced to 53% and 14% in the fibroblasts and macrophages, respectively. After 12 days, viability was reduced to 0% in both cell lines, therefore demonstrating a high degree of cytotoxicity. When PLLGA degraded for 42- and 70- days was placed in contact with the fibroblasts, cell viability reduced to 58% and 63%, respectively (Figure 4b). These values are below the 70% viability threshold that is stated by ISO 10993-5, below which the material displays cytotoxic potential. In contrast, when PLLGA was placed onto the macrophages (Figure 4b), there was no cytotoxic effect observed at any time point, with high cell viability values between 84–112%.
3.3 Cytotoxicity and Polymer Degradation

The degradation characteristics (change in pH of the degradation media, percentage mass loss, percentage swelling and change in the number averaged molecular weight (\(M_n\)) value) of PDLGA and PLLGA, degraded at 47°C, were measured[28] and compared to the MTT assay results in Figure 4. PDLGA and PLLGA degrade via a mechanism of bulk degradation, which can be observed when degradation proceeds in two distinct phases; firstly, a reduction in molecular weight, followed by mass loss. Figure 5 shows that after 5 days, there was a significant reduction in the \(M_n\) value of PDLGA from 27 800 g/mol to 3380 g/mol indicating significant change in the degree of polymerization of the polymer and break down of its chains to oligomers and monomers, with a mass loss of only 6% after 12 days. There was a slight decrease in the pH of the degradation media from pH 7.4 to 6.3 after 12 days. However, there was significant polymer swelling after 10 days (97%) and 12 days (57%), which corresponds to the reduction in cell viability in both the fibroblasts and macrophages at these time points. Figure 6 shows that the \(M_n\) of PLLGA decreased steadily with degradation time from 135 000 g/mol to 22 700 g/mol after 42 days and to 2000 g/mol after 56 days, indicating that the high molecular weight chains have almost completely broken down to shorter chains. There was initially a small decrease in mass, 2%, after 42 days, followed by a larger reduction in mass, 19%, after 56 days, and a further reduction to 38% after 70 days. There was a slight decrease in the pH of the degradation media from pH 7.4 to 6.0 after 42 days, and a significant drop to pH 2.8 after 56 days. Swelling in PLLGA did not occur immediately but increased with degradation time with a swelling ratio value of 43% after 70 days, with a corresponding cell viability of 63% in the L929 fibroblasts and 103% in the RAW264.7 macrophages.
Figure 5. Relationship of cell viability and degradation time with a) pH of degradation media, b) Mass Loss, c) Swelling and d) Mn of PDLLGA.

Figure 6. Relationship of cell viability and degradation time with a) pH of degradation media, b) Mass Loss, c) Swelling and d) Mn of PLLGA.
3.4 MTT Cytotoxicity Assay using Quasi Vivo® Flow Conditions

Figure 7 compares the viability of fibroblasts in contact with PDLLGA degraded for 10 days and PLLGA degraded for 42 days, under static and flow conditions using the Quasi Vivo® QV500 system. The samples were selected based on the results of the MTT assay, in Section 3.2, as after 10 days and 42 days, PDLLGA and PLLGA, respectively, began to show cytotoxic potential towards the fibroblasts. Figure 7a shows that PDLLGA was cytotoxic after 10 days using both static and flow conditions, with cell viability reduced to 46% and 16%, respectively. Significant differences were observed between the control and 10 days sample for both sets of conditions. PLLGA showed cytotoxic potential, under static conditions, after 42 days, with cell viability reduced to 58% (Figure 7b). However, cell viability was improved to 69% using the Quasi Vivo flow conditions and the results showed no significant difference to the control, suggesting that after 42 days degradation, the material showed less potential to induce cytotoxic effects under flow conditions than those observed under the standard static experimental conditions.

3.5 Antibody Array Analysis

Figure 8a shows the relative expression levels of the inflammatory cytokines detected in conditioned media from fibroblasts and macrophages after contact with PDLLGA degraded for 10 days. For fibroblasts, nine cytokines were detected in the control, including MCP-1, tissue inhibitor of metalloproteinase-1 (TIMP-1), macrophage colony-stimulating factor (M-CSF), stromal cell-derived factor-1 (SDF-1), keratinocyte chemoattractant (KC), interleukin-1α (IL-1α), interferon-inducible T cell alpha chemoattractant (I-TAC), tumour necrosis factor-α (TNF-α), interferon gamma (IFN-γ), and eleven cytokines were detected in the treated samples, with the addition of interleukin-17 (IL-17) and interleukin-7 (IL-7) to those already observed in the control. The results showed a downregulation in the relative expression levels of MCP-1 and KC in the treated samples. For the macrophages, eight cytokines were detected, which included MIP-1α, macrophage inflammatory protein-1β (MIP-1β), SDF-1, interferon-inducible protein-10 (IP-10), interleukin-1ra (IL-1ra), MCP-1,
TNF-α and C-X-C motif chemokine ligand 9 (CXCL9). However, only MIP-1α and SDF-1 were detected in the treated samples. The results show a pronounced downregulation of the relative expression levels of MIP-1α, MIP-1β, IP-10, IL-1ra, MCP-1 and TNF-α. Figure 8b shows the relative expression levels of the cytokines released from the fibroblasts and macrophages after contact with PLLGA degraded for 42 days. In the fibroblasts, four cytokines were detected in the control and treated samples, these include MCP-1, TIMP-1, M-CSF and SDF-1. The results show an upregulation of MCP-1, a downregulation of TIMP-1 and SDF and no change in M-CSF in the treated samples. In the macrophages, four cytokines were also detected in the control and treated samples, which include MIP-1α, SDF-1, IL-1ra and MIP-1β. The results show that there was an increase in the relative expression levels of MIP-1α, SDF-1, IL-1ra and MIP-1β in the treated samples compared to the control. In both PDLLGA and PLLGA, MCP-1 was the most abundant cytokine in the fibroblasts and MIP-1α was the most abundant cytokine in the macrophages.

Figure 8. Relative expression levels of inflammatory cytokines detected in the supernatants collected from L929 fibroblasts and RAW264.7 macrophages after contact with PDLLGA pre-degraded for 10 days and PLLGA pre-degraded for 42 days, and controls collected from the supernatants of L929 fibroblasts and RAW264.7 macrophages that had no contact with the polymers.
3.6 ELISA Analysis

Figure 9 shows the concentrations of the cytokines MCP-1 and MIP-1α, in the fibroblast and macrophage conditioned media, respectively, after contact with PDLGA and PLLGA. Concentrations of the cytokines MCP-1 and MIP-1α increased when in contact with non-degraded PDLGA and PDLGA degraded for 7 days, in both cell lines (Figure 9a). However, after 10 days, the concentration of the cytokines decreased. The concentration of MCP-1 in the fibroblasts decreased by 49% after 10 days and 28% after 12 days relative to the control. The concentration of MIP-1α decreased by 88% and 89% compared to the control, after 10 and 12 days, respectively. Statistical analysis showed that there was no significant change in the concentration of MCP-1 during the experiment, but there was a significant difference in the concentration of MIP-1α in the macrophages after 10 and 12 days.

Somewhat different trends were observed when the fibroblasts and macrophages were in contact with PLLGA (Figure 9b). The concentration of MCP-1 in the fibroblasts decreased with degradation time (Figure 9bi). There was no significant difference in the concentration of MCP-1 in the non-degraded PLLGA sample compared to the control, however, significant differences were observed after 28, 42, 56 and 70 days. There was a 42% reduction in the concentration of MCP-1 after 28 days and a 60% reduction observed after 70 days. There was no statistically significant difference in the concentration of MIP-1α in the macrophages after contact with PLLGA at any time point (Figure 9bii). However, elevated levels of MIP-1α were observed after 28 days and 70 days, with values increasing by 240% and 383%, respectively, compared to the control.

a) PDLGA

i) L929 Fibroblasts

ii) RAW264.7 Macrophages

b) PLLGA

i) L929 Fibroblasts

ii) RAW264.7 Macrophages
3.7 GPCR (HCA1) activation and calcium mobilisation assays analysis

When a ligand binds to its G-protein coupled receptor (GPCR), it undergoes a conformational change, which facilitates the activation of specific intracellular pathways. Guanosine diphosphate (GDP) bound to the Gα protein subunit is replaced by guanosine triphosphate (GTP). This initiates the dissociation of the Gα subunit from the Gβγ subunit, which in turn opens calcium ion channels and a surge of calcium is released into the cytoplasm [33]. The calcium mobilisation assay detects this influx of calcium using a fluorescent dye (Fluo-4).

This assay was used to determine whether GPCRs of the fibroblasts and macrophages, are activated in the presence of the degradation products released from PDLLGA and PLLGA during degradation in PBS buffer at 47°C. Observation of receptor activation would strongly point towards activation of the hydroxycarboxylic acid receptor 1 (HCA1), the agonist for which is lactic acid/lactate, and this is the only constituent of the degradation products known to activate GPCRs. Receptor activation is observed, in Figure 10, upon the addition of the degradation media of PDLLGA and PLLGA degraded for 12- and 70- days, respectively. The curves show a sharp increase in fluorescence, which gradually declines after reaching a maximum. This is characteristic of GPCR activation [33]. Control curves for PBS (blank) and sodium L-lactate can be found in Supplementary Information (Figure S1).
4. Discussion

4.1 Correlating cytotoxicity with degradation behaviour

The biocompatibility of bioresorbable polymers is generally tested using short-term *in vitro* cytotoxicity tests before proceeding to *in vivo* trials [34]. These studies have shown polymers composed of lactide and glycolide demonstrate satisfactory results [35,36]. Yang *et al.* [37] carried out an MTT assay on L929 fibroblasts incubated for up to 7 days with extract medium prepared using PDLLGA (70:30). The results showed no cytotoxic effects at any time point. However, this study did not account for how polymer cytotoxicity may change during a more prolonged degradation timeframe. Therefore, long-term cytotoxicity studies are needed to assess the effects of these polymers and their degradation products in the physiological environment. Previously, Sung *et al.* [38] compared the biocompatibility of the fast degrading PDLLGA to slow degrading poly(ε-caprolactone) (PCL), by culturing mouse aortic smooth muscle cells on PDLLGA and PCL polymer scaffolds and assessed the *in vitro* cell viability after degradation for 1, 7, 14, 21 and 28 days at 37°C. The results showed that the cell viability in the fast-degrading PDLLGA was lower than that of the slow degrading PCL. It was suggested that the reduction in cell viability was due to the increase in acidification of the local environment during polymer degradation. Despite the results showing differences in the cytotoxicity of the two polymers, the study did not consider that the fast-degrading PDLLGA was significantly more advanced in terms of the relative course of its degradation, than the slow degrading PCL after the 28 day period. For a true comparison, it is important to evaluate the polymers at similar levels of degradation, considering for example, mass and molecular weight loss. However, the slow polymer degradation times at 37°C can make it difficult and impractical to obtain polymer samples for study at adequate extents of degradation. One method to achieve this is to degrade the polymers at an increased temperature. Ignatius *et al.* [39], evaluated the biocompatibility of poly(D,L-lactide) (PDLLA) 70:30 and PLLGA 90:10 using a series of *in vitro* tests, including an MTT assay on BALB 3T3 cells. The MTT assay was performed using the extracts of PDLLA and PLLGA prepared in PBS buffer for 10 days at 37°C and 70°C. The extracts prepared at 70°C were used to simulate the degradation products released after the long-term degradation of the polymers. To exclude pH concerns during the assay and to focus on the degradation products, samples were neutralised to pH 7.4. The results of the assay found that cell proliferation was significantly reduced when in contact with high concentrations of the extracts prepared at 70°C. Although the conclusions of the study found both polymers showed acceptable biocompatibility overall, it was suggested that high concentrations of degradation products were toxic to the cell lines.

*In vitro* cytotoxicity tests are carried out by placing the test material or extract solution directly onto the cultured cells [34]. Extract tests are useful in determining the cytotoxicity of leachable substances released from the biomaterial, however, they do not provide any information on how a material interacts directly with the cells. To the best of the authors knowledge, no *in vitro* study has demonstrated how the cytotoxicity of polymers composed of lactide and glycolide change during degradation, using a direct contact test. Direct contact tests are highly sensitive and can be used to detect even small levels of cytotoxicity in samples. One limitation of direct contact studies is the potential for mechanical damage to the cell monolayer, therefore great care must be taken with experimental technique when placing the material in contact with the cells. To overcome this, we have developed a protocol for pre-degrading polymers while cradled within a transwell insert, which is then transferred directly from degradation media to cell culture. This method has been validated against conventional direct contact methodology, showing no significant difference in results, but with the advantage of convenience during sample handling, especially for degraded samples that...
become friable or gel-like, and that are difficult to keep intact on handling (Supplementary information Figure S2). The aim of this study was to develop an in vitro protocol that can be used to assess the cytotoxicity of bioresorbable polymers, at different stages of degradation, in an accelerated period. The increased temperature accelerated degradation methodology, validated in a previous study [28], was used to assess the long-term cytotoxicity of PDLLGA and PLLGA. Both polymers were pre-degraded in PBS buffer at 47°C, before being placed on L929 fibroblasts and RAW264.7 macrophages.

The results of this work demonstrate differences in the cytotoxic potential of PDLLGA and PLLGA. The cytotoxicity of PDLLGA increased significantly at the later stages of degradation, after 12 days with 0% cell viability in both the fibroblasts and macrophages, whereas PLLGA demonstrated low levels of cytotoxicity against fibroblasts after 42 and 70 days, with 61% and 64% cell viability, respectively. The correlation of these results to the degradation behaviour data is shown in Figure 5 and Figure 6. PDLLGA showed a significant reduction in cell viability, in both fibroblasts and macrophages, at the later stages of degradation. Figure 5 shows that there were no significant reductions in the pH of the degradation media or mass of the polymer after 12 days. However, a large reduction in the $M_n$ was observed after 5 days, with substantial polymer swelling occurring after 10 days. This indicates that low molecular weight polymer chains are being trapped within the polymer matrix. It is likely that these acidic degradation products are released from the polymer during incubation with the cells, overwhelming the local microenvironment at the polymer/cell interface and causing a significant reduction in cell viability. In comparison, PLLGA did not become toxic to the macrophages but showed cytotoxic potential against the fibroblasts after 42 days and 70 days. As shown in Figure 6, there was no significant mass loss after 42 days, but there was a gradual decline in the molecular weight of the polymer. This suggests that low molecular weight chains are retained within the polymer matrix. These acidic chains are subsequently released during incubation with the fibroblasts, causing a reduction in cell viability. After 56 days, mass is lost from the polymer and a significant decrease in the pH of the degradation media demonstrates the release of the acidic degradation products from the polymer matrix. Therefore, when this sample is in contact with fibroblasts, there is no reduction in cell viability as there is no accumulation of acidic degradation products within the polymer matrix. After 70 days, further mass loss occurs, however, there is an increase in polymer swelling. Swelling occurs via an influx of the acidic degradation media, and a reduction in cell viability occurs when the acidic degradation products are released from the polymer matrix upon incubation with the fibroblasts. It is unclear why the cytotoxic effect was only observed in the fibroblasts and not the macrophages. However, it should be noted that the cytotoxic effect observed for PLLGA was significantly lower than that of PDLLGA, which suggests PDLLGA has greater potential to display cytotoxicity compared to PLLGA. The molecular weight distributions, from a previous study by Geddes et al.[28], revealed that PDLLGA is more acidic than PLLGA at the later stages of degradation as high amounts of oligomers and monomers are retained within the polymer matrix of PDLLGA. This could explain the differences in cytotoxicity of the two polymers.

The temperature accelerated degradation methodology validated in a previous study [28], can be used as a method to pre-degrade specimens at the accelerated temperature (47°C) where physiological temperature (37°C) would be impractical due to the time duration required. It should be noted however that the model is only valid up to the point where mass loss occurs, so beyond this point it will become a less reliable tool. The model developed found that the rate of hydrolysis at 47°C was increased 3.7-fold in PDLLGA and 21.9-fold in PLLGA compared to 37°C. Polymer degradation time is dependent on many factors, such as geometry, composition, crystallinity and implant location. It can, therefore, be difficult to give exact degradation times for every scenario and it is acknowledged that, in the in vivo environment, factors such as phagocytosis combined with
enzymic action may play a role in the later stages of degradation [40]. Few \textit{in vivo} animal studies or indeed clinical studies have monitored PLLGA degradation up to the point of full resorption. Of four studies identified for PLLGA \textit{in vivo} degradation, none continued to monitor up to full resorption, so there is no definitive answer as to when this occurs. What can be determined from the literature [40–43], is that PLLGA is likely to be substantially resorbed within 24 months \textit{in vivo}. It should be noted, however, that additives such as ciprofloxacin [40] or tricalcium phosphate (TCP) [41,43] may influence polymer degradation rate. Our previous work, using an \textit{in vivo} rabbit model, showed a slight, but non-significant reduction in degradation rate in PLLGA screws loaded with TCP, compared to screws composed of PLLGA polymer alone [47].

Throughout the literature, an inflammatory foreign body reaction for PDLLGA has been observed to occur between 14–243 days [21,41,44–48]. Fewer incidences of foreign body reactions have been reported for PLLGA, with some reporting no foreign body reactions [40,42], and others reporting incidences of cyst formation occurring between 730–1460 days [41,43]. Based on the results of this \textit{in vitro} study, the degradation times extrapolated to 37°C, indicate adverse reactions would be predicted to occur 37 days after implantation for PDLLGA and after 920 days for PLLGA. This extrapolation method corroborates the results observed in this study with the adverse reactions noted by clinical case reports in the literature [21,41,43–48].

4.2 Development of an improved method to test for cytotoxicity

In Section 4.1, fibroblasts and macrophages were cultured in traditional static 24-well plates. An MTT assay was carried out, after PDLLGA and PLLGA were placed on the cultured cells and incubated for 24 hours. Multiwell plates, used in this way, have become a standard in cell culture experiments. However, static conditions are limited as they do not resemble the dynamic nature of the physiological environment. The QV500 system was investigated as a technique that could be used to improve upon the static multiwell plate conditions, and better mimic \textit{in vivo} conditions. The transwell methodology used for the static conditions was adapted using the QV500 system, as the dimension of the chambers (15 mm internal diameter, 10 mm height) are similar to those of the standard 24-well plate [49,50]. The system was connected in series to allow for a continuous flow of media through each chamber. Therefore, degradation products, released into the media, are continuously carried away and replenished with fresh media, as would be the case \textit{in vivo}, where degradation products would diffuse from the vicinity of the implanted material following a concentration gradient, and where there would be a continuous exchange of tissue fluid. In this study, samples were assessed for cytotoxicity using the QV500 system and compared to the response observed under static conditions (Figure 7). The results demonstrate that when PDLLGA reaches a certain stage of degradation it becomes inherently cytotoxic to the fibroblasts, even under flow conditions. This suggests that the amount of acidic degradation products released from PDLLGA exceeds the clearing capacity of the flow system, resulting in a reduction in cell viability. PLLGA showed improved cell viability using the flow system with no significant difference between the degraded sample and the control, whereas under static conditions a cytotoxic response was observed. This indicates that the flow of fresh media through the chamber provides sufficient clearance of the acidic degradation products released from PLLGA. The flow system is more indicative of the true \textit{in vivo} scenario where diffusion through, and replenishment of tissue fluid in the peri-implant environment will result in the clearance of acidic degradation products.

Throughout the literature there are reports that implanting polymers composed of lactide and glycolide in bone regions with poor vascularity can result in an adverse reaction occurring
This study has demonstrated that methodology using flow conditions can reveal an improved cytotoxicity profile of one polymer (PLGA) but have no impact on another (PDLLGA). Polymers in the poly(lactide-co-glycolide) family degrade via heterogeneous bulk degradation. However, each polymer within this family exhibits different degradation characteristics which are dependent on a number of factors, such as polymer morphology, composition and configurational structures, and the geometry of the device [53,54]. Future research should consider how different polymer degradation mechanisms and characteristics will result in differences of physiological response.

4.3 Further Evaluation of the Inflammatory Response

The aim of the final stage of this study was to gain a better understanding of the in vitro inflammatory response of fibroblasts and macrophages to PDLLGA and PLLGA at progressive stages of degradation. The results of the antibody array (Figure 8) and ELISA (Figure 9) demonstrate a general downregulation of inflammatory cytokines at degradation levels where PDLLGA and PLLGA begin to show cytotoxic potential. Alternatively, when no cytotoxicity was observed, there was either no significant change or an upregulation in the expression of the inflammatory cytokines. This was contrary to our expectations, as it was anticipated that materials displaying cytotoxic potential would initiate an inflammatory response. Surprisingly, these results suggest an immunosuppressive or anti-inflammatory effect of PDLLGA and PLLGA. Sung et al. [38], compared the biocompatibility of fast degrading PDLLGA to the slow degrading PCL. The polymer scaffolds were implanted subcutaneously in wild-type 129/SvEv mice, harvested after 7, 14, 21 and 28 days and assessed for the density of inflammatory cells within the polymer. It was found that a significantly reduced number of inflammatory cells migrated into PDLLGA than PCL and it was suggested that the acidic environment, created by PDLLGA, inhibited cell migration. However, it is not clear what inflammatory cells were detected. Britland et al. [55] found a dose dependent relationship between lactate concentration and cell viability using an MTT assay. It was observed that cell viability was reduced when cells were treated with a lactate concentration of 10 mM for human umbilical vein endothelial cells (HUVEC) and between 10 and 50 mM for 3T3 fibroblasts. However, the authors were not able to determine the concentration threshold for when lactate made this change from being a positive influence on the healing process to becoming a detrimental presence.

In light of our results discussed above, we formed the hypothesis that the degradation products of PDLLGA and PLLGA must be active in an immunomodulatory capacity. Lactate/lactic acid, a major degradation product, has been found to be biologically active and involved in the signalling pathways and regulatory functions in many physiological and pathological conditions [56,57]. Lactate is the conjugate base of lactic acid and is formed in the body as the end product of anaerobic or aerobic glycolysis [55]. Anaerobic glycolysis yields lactate under hypoxic conditions in normal cells, whereas, in aerobic glycolysis (Warburg effect [58]) lactate is produced, in the presence of oxygen, in rapidly dividing cells, for example, in cancer cells or during wound healing [56,59]. Monocarboxylate transporters (MCTs) are used to transport lactate across cell membranes, which is carried out by a series of reactions, involving the conversion of lactate to pyruvate and alterations to the intercellular NADH/NAD⁺ ratio [59,60]. Lactate has traditionally been considered as an energy-rich metabolic fuel, but more recently its role as an important cell signalling molecule has been come to light. It was observed for the first time in 2009 that HCA₁ (also known as G-protein coupled receptor-81 or GPR81) could be activated by L-lactate [56,57,59,61]. The D-lactate enantiomer has also been reported as a agonist/partial agonist of the receptor [61–63]. The stimulation of HCA₁ by lactate results in cell signalling by the downregulation of cAMP and the activation of non-canonical, β-
arrestin-dependent signalling pathways [56,57]. This method of signalling is different to MCT-driven transport and its discovery has led to significant interest in the role of lactate as a cell signalling molecule through the activation of HCA_2. Lactate has been shown to have an important role in the modulation of the immune response and inflammation of various pathophysiological conditions. The activation of HCA_2 has been shown to have an immunosuppressive effect on the regulation of intestinal inflammation by signalling the downregulation of inflammatory cytokines in colonic macrophages and dendritic cells [64]. The presence of lactate generated via aerobic glycolysis in cancer cells has been found to promote tumour growth and metastasis by acting as an immunosuppressant [59,65,66]. Lactate has also been shown to increase collagen deposition and promote angiogenesis by inducing the release of proangiogenic factors, such as VEGF [67–70]. The therapeutic effect of poly(lactide-co-glycolide) (PLGA) polymers in wound healing and as a drug delivery system has been reported in the literature. Porporato et al. [69], reported that wound healing was accelerated in two strains of mice, by delivering a rate-controlled release of lactate using PDLG 50:50. It was found that a nearly 60% decrease in excisional wound closure, 10 days post injury, and a 2.2-fold increase in angiogenesis in the mice treated with the polymer occurred. Similar results were also observed by Chereddy et al. [71], with a 75% wound closure observed after 10 days, when the injury was treated with 1 mg of PLGA nanoparticles, however, it is unclear which enantiomer of lactide was used. They also observed a downregulation in the inflammatory expressions of glutathione peroxidase (GPx) and nuclear factor-κB (NFκB). These observations reported in the literature, and the results reported in this present study (inflammatory cytokine profiling, and media from PDLGA and PLLGA degradation studies inducing GPCR receptor activation, as demonstrated by calcium mobilisation), have important implications for lactic acid-based biodegradable materials used for medical devices, such as orthopaedic fixation screws. These devices have to date been chosen for their physical and mechanical characteristics, however their effects on host tissues may be much further reaching, given the proven cell signalling role of lactate. Devices made from these polymers have the potential to be bio-active and influence inflammation and healing at the site of implantation.

The results in this study support what has been revealed in the literature in relation to the immunosuppressive effect of lactate. During the degradation of PDLGA and PLLGA a downregulation in the inflammatory cytokines in fibroblasts and macrophages was observed. The results from the calcium mobilisation assays demonstrate that this observed effect is likely to be due to the activation of the GPCR, HCA_2, by the polymer degradation product, lactate/lactic acid, which in turn acts as a signalling molecule to downregulate the cytokines. This effect is likely to have an impact on the in vivo implantation of PLGA and PLA polymers. It is possible that as a polymer degrades, in vivo, the lactate released initially has a therapeutic anti-inflammatory effect on the surrounding tissue. However, at the later stages of degradation, significant polymer erosion leads to a large release of acidic degradation products, which could be considered the ‘tipping-point’ at which lactate levels overwhelm the polymer/tissue environment.

5. Conclusion

The results observed in this study demonstrate the occurrence of a delayed inflammatory reaction at the late stages of polymer degradation. It can be difficult to extrapolate in vitro results to in vivo applications as the conditions are very different. However, the use of the increased temperature, 47°C, to accelerate the degradation of PDLGA and PLLGA has demonstrated great potential for the assessment of biodegradable polymers with long degradation times as the results extrapolated to 37°C corroborate with the clinical data of adverse reactions occurring, in vivo, in the literature.
Efforts were also made in this work to improve upon the static conditions generally used for in vitro research. The results validate the feasibility of using the QV500 system as a method to better represent the in vivo flow environment. This study has shown differences in the cytotoxicity of PDLLGA and PLLGA and proposed explanations for the observed response. It is recommended that future research into orthopaedic devices focuses on PLLGA.

The final section of this work demonstrates the immunosuppressive and anti-inflammatory role of lactate. To the best of the authors knowledge, the effect of lactate in the clinical outcomes of PLA and PLGA used for orthopaedic devices has not been considered in the literature, due to the recent discovery of the ligand activity of lactate at HCA1 receptor. It is likely lactate plays a significant role in the cell signalling and regulatory functions at the site of polymer implantation. It is therefore important future research considers this effect when analysing the biological response of these polymers.

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