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Polylactic is a sustainable, low absorption, low auto-fluorescence, alternative to other plastics for Microfluidic and Organ-On-Chip applications

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Supporting Information Placeholder

ABSTRACT: Organ-on-chip (OOC) devices are miniaturised devices replacing animal models in drug discovery and toxicology studies. The majority of OOC devices are made from Polydimethylsiloxane (PDMS), an elastomer widely used in microfluidic prototyping, but posing a number of challenges to experimentalists, including leaching of uncured oligomers and uncontrolled absorption of small compounds. Here we assess the suitability of Polylactic Acid (PLA) as a replacement material to PDMS for microfluidic cell culture and OOC applications. We changed the wettability of PLA substrates and demonstrated the functionalization method to be stable over a time period of at least 9 months. We successfully culture human cells on PLA substrates and devices, without coating. We demonstrated that PLA does not absorb small molecules, is transparent (92 % transparency) and has low autofluorescence. As a proof of concept of manufacturability, biocompatibility and transparency we performed a cell tracking experiment of prostate cancer cells in a PLA device for advanced cell culture.

KEYWORDS: Microfluidic cell culture, Organ-On-Chip, Poly-Lactic Acid (PLA), transparency, biocompatibility, rapid prototyping, cell tracking

INTRODUCTION

Organ-On-Chip (OOC) is the convergence of two emerging research areas, microfluidics and tissue engineering, meeting the requirements for more physiologically accurate human tissues. OOC devices provide advanced models for cell and tissue culture, accelerating and facilitating the understanding of human biology, toxicology, disease progression and prognosis, while overcoming important ethical, financial and interspecies variances related to animal experimentations.[1]

Since the birth of the first OOC device in 2007 [2], complex functionalities have been added to devices, leading to the so-called body-on-a-chip or human–on-a-chip fields. The translation from the research to the market was relatively fast. To date, at least 28 companies are proposing body and tissue on-a chip devices [3]. The material of choice in most organ-on-chip platforms has been poly(dimethylsiloxane) (PDMS), a thermo-curable elastomer. The majority of companies present in the OOC market (57%) sells PDMS products (Suppl. Inf. 1). Although PDMS is biocompatible, transparent, gas permeable, flexible, and relatively easy to manufacture at small scale, some issues have been encountered by consumers while using PDMS devices for certain applications. These problems include channel deformation, high evaporation, leaching of uncured oligomers, absorption of hydrophobic compounds and unstable surface treatment which can lead to inconsistent and unpredictable results with respect to some biological outcomes [4]. While some of these issues have been overcome (e.g. via soxhlet extraction in ethanol, or other organic solvent, to remove uncrosslinked oligomers [5]), PDMS moulding still remains a difficult process to fully automate [6] and significantly slows down the translation from the research to the mass market production.

The need for alternative materials to PDMS is such a concern for the industry, that the US Small Business Innovation Research, SBIR, has recently funded studies to find non-PDMS alternatives, providing specific requirements to be addressed, e.g. transparency, biocompatibility, no absorption and manufacturability.

Poly methyl methacrylate (PMMA), Polycarbonate (PC), Cyclic olefin polymers and copolymers (COP and COC respectively) and Polystyrene (PS) are some common materials proposed from different suppliers as scalable alternatives. These thermoplastic materials present the advantage of being relatively cheap, and could translate easily to the mass market. However, these materials do not always exhibit good biocompatibility and are fossil-based, therefore unsustainable.

In a recent study, we explored the mechanical properties of Polylactic acid (PLA), a biocompatible thermoplastic material derived from renewable resources as an environmental friendly substrate material for the production of environmentally sustainable, single-use microfluidic devices adopting a design for sustainability approach [7]. PLA has been widely studied in the context of tissue engineering, and in approve by the FDA as a drug carrier. Evidence of PLA suitability for advanced microfluidic 3D cell culture have been shown previously [8]. Additionally, PLA is compatible with high volume production such as microinjection moulding. PLA is also easily workable and compatible with rapid-prototyping. For example, a number of researchers have manufactured PLA-based devices using 3D-printing. 3D-printed PLA structures are often non-transparent and demand post-processing. On the other hand, PLA can be formed into sheets, machined by laser cutting or milling, integrated to other structures such as electrodes or membranes and assembled into complex microfluidic devices [7].

Despite the increasing use of PLA in the field of microfluidics, no characterization and assessment of transparency, autofluorescence, absorption of small molecules and comparison with other thermoplastic have been carried out on PLA. Here we provide evidence that PLA is a suitable replacement to other polymers in Organ-on-a-chip applications, readily to be translated to the mass market. We investigate surface properties, biocompatibility, absorption of hydrophobic and hydrophilic small compounds (< 900 Da), and optical properties of PLA in comparison to PDMS and with other reference polymers such as PS. Finally, we provide an example of cell-tracking through a PLA-based microfluidic device.

METHODS

Materials

Polylactic acid (Natureworks® 2003D) was purchased from Naturework in pellet format. PMMA Clarex® was purchased from Weatherall Ltd, in a 1 mm sheet form. PDMS Sylgard 184 was purchased from Dow Corning, while COC (Topas), PC (RS components) were acquired in 1 mm sheet. All the reagents used were purchased from Sigma Aldrich, unless specified in the text.

PLA Functionalization

The functionalisation process was an alkaline surface hydrolysis. NaOH was dissolved in DI water to prepare 0.01, 0.1, 0.5, and 1 M solution concentrations. The pristine PLA, pPLA, sample was dipped inside the solution and kept in for specified amount of time (0, 10, 30, 60, 120 sec). Then the sample was washed with water and dried with compressed air. To measure the effect of the functionalization time, and solution concentration, the contact angle of the samples was measured using a custom-made static contact angle apparatus [9]. The images acquired via a Dinolite digital microscope (Dino-Lite Premier2 AD7013MZT) were analysed using the image analysis freeware ImageJ (plugin contactj). Each measurement was carried out 6 times. To assess the chemical changes at the surface an attenuated total reflection, ATR-FTIR, analysis was carried out collecting 200 scans in the range of 4000-400 cm-1, with a resolution of 4 cm-1 using a Nicolet Is5 spectrophotometer. The effect of the surface functionalization on the surface were studied using a white light interferometer (Zygo) with 1 nm resolution in vertical direction. The Roughness (Ra) quantitative determination was carried out using Metro.Pro 8.2. For morphology analysis SEM (Quanta FEG 650 SEM) images were acquired in high vacuum mode.

PLA Biocompatibility

Biocompatibility of PLA was tested by culturing human hepatoblastoma C3A cell line, A549 cells and human umbilical vein endothelial cells (HUVECs). We compared the biocompatibility of PLA with other common thermoplastic material used for microfluidic cell culture and with PDMS. Cell survival in a PLA cell culture microfluidic device was tested with HUVECs and compared with respect to a PDMS

device. Details regarding the cell culture, staining process can be found in the Supporting Information S3.

Finite Element Analysis

Finite element analysis was conducted using FEATool MultiphysicsTM version1.10 (Matlab) to determine the oxygen concentration level inside the microfluidic cell culture chamber. A simplified 2D model of the PLA microfluidic device was designed. The Oxygen diffusion coefficient of 2.5x10-12, 3.5x10-9, 4x10-9 and 2.14x10-5 m2/s was used for PLA, PDMS, water and air respectively, taken from previously published studies [10,11]. Details regarding the numerical simulations can be found in the Supporting Information S3

Microfluidic device preparation

To manufacture the PLA Organ-On-Chip devices, firstly PLA sheets were manufactured using a manual hydraulic heated press as per method described in [7]. Then each layer was cut using a CO_2 laser cutter (Epilog Mini Elix 30W) using SLAM approach, which consists of applying a thin tape (100 µm) on the material prior to laser-cutting [7]. The use of a commercial CO_2 laser enables the formation of channels with 200 µm minimum size, however, other types of laser could deliver smaller channel widths. Some examples are discussed in Figure S4. The layers were cleaned by sonicating in pure Ethanol. The cleaned layers were functionalized by dipping in a 1M NaOH solution for 60 s, then cleaned with water, 2-Propanol and compressed air. The surfaces were then chemically activated under UV exposure (254nm) for 45 s. Layers were bonded together placing them in a sandwich composed of two glass slides, and kept them in contact at 50 °C for 10 minutes. Once the assembly has cooled down to room temperature, it is disassembled and the device is ready to be tested.

Small molecules absorption

To assess the bio-inertness of the material, the prinstine PLA (*p*PLA) was compared with functionalised PLA (*f*PLA), and with PDMS. To test the absorption of hydrophobic compound Nile Red was dissolved in EtOH 99% to obtain a solution concentration of 1 µM. To test the absorption of hydrophilic compounds fluorescein salt was dissolved in DI water (1uM). A fluorescent microscope (AM4115T-GFBW) was used to take the fluorescent images and the image analysis via ImageJ. Details of the manufacturing protocol can be found in the supplementary information S5.

PLA Optical properties

The transparency of the materials was tested by analysing the transmittance of the light through the samples in the visible region using a UV-VIS spectrophotometer (Shimadzu UV-2550). The autofluorescence of the plastic materials of interest was measured using a Leica SP8 3X STED laser scanning microscope equipped with two Internal Spectral Detector Channels (PMT). More details of the protocol used to measure material autofluorescence can be found on supplementary information S6.

Cell imaging and tracking on a PLA device

A small-scale inverted microscope suitable to work in high humidity environments (incubators) with factory standard optics, custom aluminium structure and fully sealed electronics was used to perform cell tracking. The video sequence is then processed by a proprietary software (Cell-Hunter) elsewhere validated in cancer immune interaction and block replication in prostate cancer cells[12]. For the sake of comparing the kinematic characteristics of cells involved in the competing experiments, we extracted standard descriptors such as average speed, curvature, and angular speed, along with the coefficient of diffusion. The distribution of such descriptors was computed over all the tracks extracted. Details of the cell imaging and tracking experiment can be found in the supplementary information S7.

RESULTS

PLA functionalisation

An OOC device provides support for tissue attachment and organization to simulate organ-level physiology. The first interaction between the cells and the device happens at the material surface. The requirements for a suitable substrate material for healthy cell environment and tissue adhesion, are wettability, surface roughness and chemical composition[13,14]. PLA is a hydrophobic polymer, which is a limiting factor for cell attachment. To address this, surface treatments are required to increase the substrate wettability [15,16]. The most widely utilised and preferred surface modification methods rely on oxygen plasma and UV-ozone treatments [17]. However, several studies have reported the non-permanent nature of those functionalization methods, leading to the short term recovery of the hydrophobic property [18]. Such a phenomenon is particularly exhibited by PDMS, with a drastic increase of the contact angle one week after the treatment [18]. To overcome the hydrophobic recovery issue, PDMS devices need to be used just straight after the functionalisation process, which further limits their industrial applications. The contact angle value gives a quantitatively measure of the hydrophilic or hydrophobic nature of a surface. In particular, a material is hydrophilic if it has a contact angle below 90°, and hydrophobic if the contact angle is above 90°. Our PLA sheet manufacturing protocol produces a slightly hydrophilic surface with a weak wettability. The measured contact angle is about 77-80°(Fig. 1A), while the desired contact angle for cell culture applications is 45°±4° (appropriate contact angle to avoid electrostatic interactions with hydrophilic molecules [18]). In order to permanently modify the surface properties of *p*PLA, a wet-chemistry approach was adopted(Fig.1A). Using this functionalisation process, the final contact angle can be tuned by changing the functionalisation time and the solution concentration (Fig. 1B). This functionalisation protocol is stable over time, with no evidence of a hydrophobic recovery effect, even 9 months after the functionalization, at room temperature storage (Fig. 1C.i). In accordance with previous results reported by Tham et al. [19], this method acts on hydrolysing and eroding the material surface, inducing a visible change in the surface roughness (Fig. 1C.ii-D-E). In conclusion, the proposed functionalisation protocol produces the desired contact angle for cell culture. The resulting formation of the -COOH and of -OH groups at the surface can be readily used for further conjugation of surface modifying species [16,19].

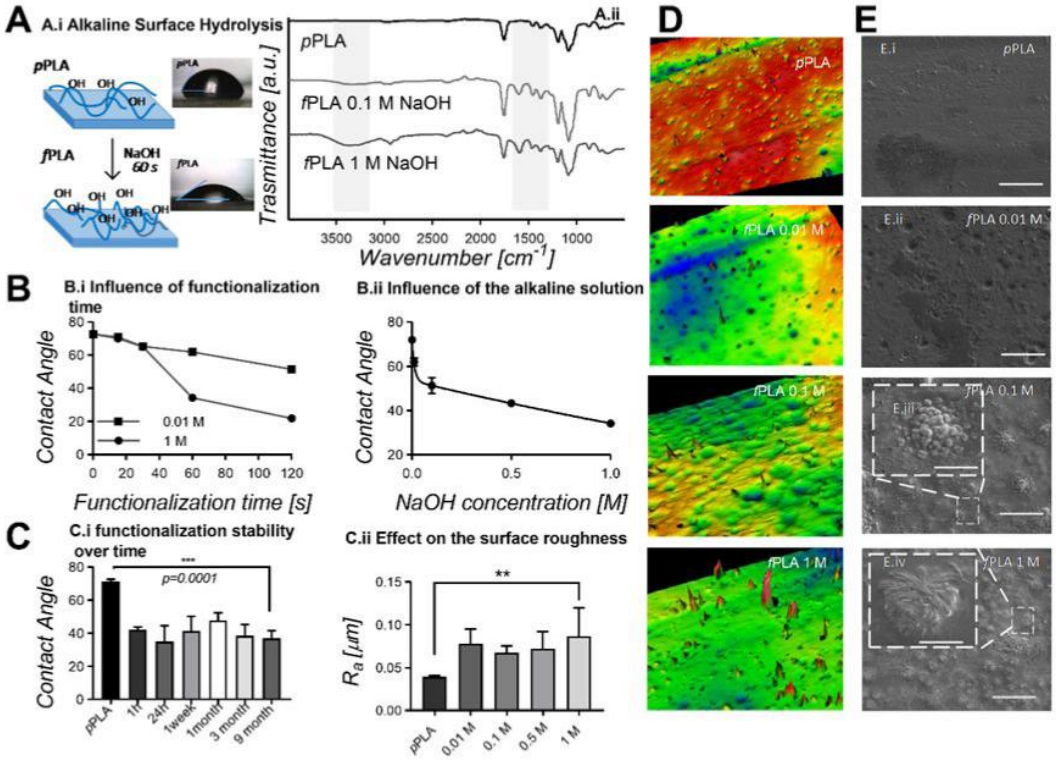


Figure 1 Tuning the surface properties of PLA. A.i) Functionalization mechanism via Alkaline Surface Hydrolysis and photographs of a DI water drop on the pristine PLA substrate (top) and on the functionalized PLA substrate (bottom) A.ii) FTIR spectrum of pristine PLA (black curve), functionalized PLA using a concentration of 0.1 M of NaOH solution (light grey curve), and a concentration of 1 M of NaOH (grey curve). The grey areas underline the areas where a change in the spectrum is observed due to the functionalization. The first one (from left to right) at 3200 cm-1 is indicative of the formation of hydroxyl groups, the second one highlights a stretching of the C-C groups indicative of a partial hydrolysis of the treated surface. B.i) Influence of the functionalization time on the final contact angle for two different solution concentration, 0.01 M (box) and 1 M (circle). The two connecting lines are just to guide the eye. For all the points the error bars are shorter than the height of the symbol. B.ii) Influence of the NaOH concentration on the final contact angle after 60 s of functionalization. The experimental results are fitted with a two-phase exponential decay function. For some points the error bar is shorter than the high of the symbol. C.i) Stability of the functionalization method adopted over nine months. C.ii) Effect of the solution concentration on the final surface roughness of the functionalized substrate after 60 s functionalization. D) Interferometer pictures of the unfunctionalized and funcionalized PLA surfaces with different solution concentration after 60 s functionalization time. Interestingly, a pillar formation structure is noticed with an increase of the pillar density with respect to the solution concentration. While increase of surface roughness is visible on SEM imaging, the pillar formation is partly damaged by the conductive gold layer. E) SEM pictures of the unfunctionalized and functionalized PLA surfaces with different solution concentration after 60s functionalization time. Atomic Force Microscope Imaging of Pristine and Functionalized PLA was also used to confirm the pillar formation. Data shown in Figure S2.

PLA biocompatibility

Biocompatibility in OOC devices can be defined as the absence of toxic effects to the cells or tissue interacting with the material, as well evidence of optimal growth of the cultured cells or tissues. PLA has been widely used in medicine as and its biocompatibility has been well established by a number of studies, typically demonstrating lack of low inflammation after implantation and compatibility with surrounding tissue [20,21,22]. In this study, we specifically aimed at comparing the biocompatibility of pristine PLA (*p*PLA) versus functionalised PLA (*f*PLA), as well as against PS (control material), and PDMS. In supplementary information 3 (S3), detailed material and methods are given, as well as a comparison with other common thermoplastic materials. Cell proliferation and cell death of two cell lines, human hepatocellular carcinoma cells (C3A) and adenocarcinomic human alveolar basal epithelial cells (A549) were examined. At 48 hrs of cell culture of the C3A cells, only the substrates made of *p*PLA, *f*PLA and *f*PDMS performed comparably with the control (albeit the differences with other polymers were small, figure S3), and at 7 day time point *p*PLA and *f*PLA showed a decrease in cell death of 6% and 10% respectively, with respect to the control (Fig. 2 A.i). For the A549 cells at 48 hrs of culture almost all the substrates performed comparably well to PS control with no significant statistical difference in the cell death, while at 7 days all polymers caused the same percentage of cell death as compared to control with the exception of PLA that demonstrated a decrease in cell death (Fig S3). Cell proliferation data is shown in Figure S4.

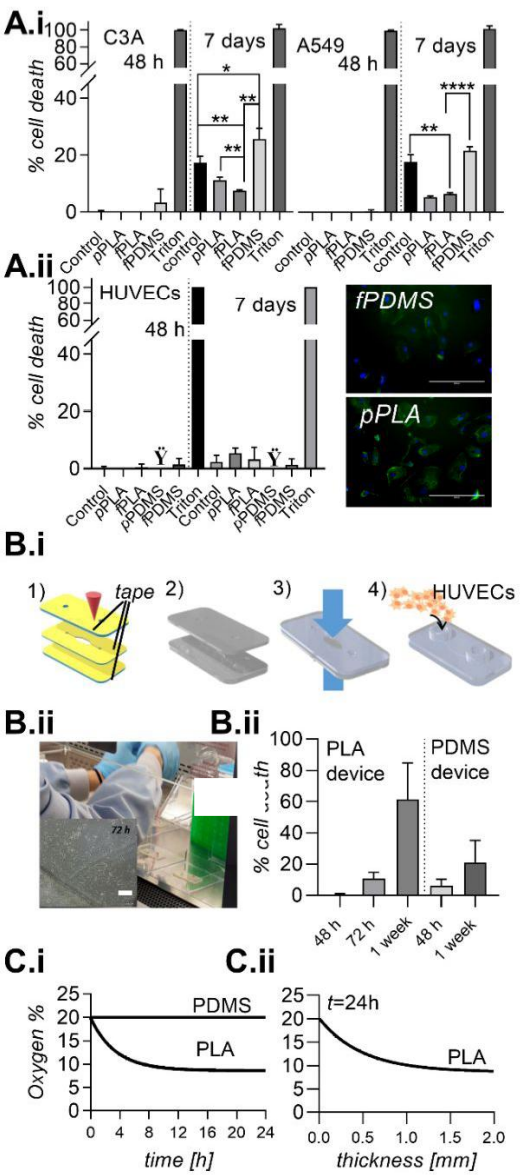


Figure 2 A) Assessing the material biocompatibility off-chip. Percentage of cell death of C3A, A549 (A.i) and HUVEC cells (A.ii) at 48 h and 7 days time points. For detailed method see S4. The positive control (noted Control) is uncoated PS wells. 0.1% Triton X-100 (Sigma, UK) (Triton) is a negative control, in uncoated PS cells, provides 100% cell death comparison. HUVEC cells did not adhere on the pristine PDMS (Noted with a symbol: Ÿ). In Inset of A.ii, DAPI-actin staining of HUVECs at 7 days time point on fPDMS and PLA(scale bar is 200 µm). B) Cell culture on-chip B.i) Schematization of the protocol to manufacture the microfluidic device for culturing HUVECs, 1) CO_2 laser cutting the three layers using the SLAM approach; 2) Cleaning and functionalizing the layers; 3) UV bonding the layers; 4) Device ready to be sterilized and used. B.ii) Photograph showing the cells being loaded inside the device. In insert, bright-field microscope photograph of healthy HUVECs cells with good confluence after 72h culture, scale bar is 200 µm. B.iii) Comparison of HUVECs cell death in the PLA and in the PDMS device. C) 2D Numerical simulation of oxygen level inside the microfluidic culture chamber. C.i) Simulated oxygen levels in function of culture time inside the PDMS and PLA microfluidic device. The oxygen level in the PLA level quickly reduces to 10% after a few hours C.ii) Simulated oxygen level in function of different thicknesses of PLA device sealing layer at 24h. A thickness of 0.250 mm would enable oxygen levels to be within 5% of the atmospheric level after 24h

We also independently investigated the PLA biocompatibility with primary cells, which have a finite life span and a limited expansion capacity, compared to cancer cell lines. We cultured freshly isolated human umbilical vein endothelial cells (HUVECs) for 7 days on the same substrates Both *p*PLA and *f*PLA performed as the control PS (no statistical difference). Pristine PDMS (pPDMS) did not allow cell adhesion due to the intrinsic hydrophobic properties of the material (Fig. 2 A.ii). Overall, the toxicity data of cancer and primary cells, indicates that PLA is a suitable material for cell culture. Translating from a PS substrate to a PLA substrate was found to be marginally better for A549 and C3A cells, and similar for HUVECs.

To further evaluate the biocompatibility of *f*PLA and its suitability as substrate material for microfluidic cell culture and OOC application, a 3 layer microfluidic device consisting of a single cell culture rectangular chamber was fabricated as per material and method session (Fig. 2 B.i). Primary HUVECs were cultured for one week to evaluate morphology and proliferation. The material transparency and absence of autofluorescence enabled to easily evaluate cell viability using the ReadyProbes® kit. (as shown in inset of Fig. 2 B.ii) and the cells easily adhered onto the PLA microfluidic walls without the need for adhesive protein coating. They formed a confluent layer after 3 days in culture, maintaining their natural morphology. We compared the performance of the PLA device with an identical single chamber PDMS device (Fig 2.B.iii). No statistically significant differences were noticed in the culture of the cells between the two different substrate materials, up to 72 h. At 1 week time point a significant increase in cell death was noticed inside the PLA device. This is likely to be related to the lower oxygen permeability of PLA with respect to PDMS, static condition of the culture (media changed every 24h) and the increasing oxygen demand due to cell proliferation [23]. In order to evaluate the oxygen concentration inside the PLA cell culture chamber and compare it to a PDMS chamber, we used FEATool Multiphysics, a Matlab toolbox to create a 2D model. A time dependent condition was imposed and the oxygen concentration simulated over 24h with a time step of 1h assuming an oxygen consumption rate of the cells of 0.37×10^{-4} mol/s [24] (Fig 2.C.i). We carried out different simulations by changing the thickness of the top sealing layer and plotted the oxygen concentration level at 24 h time point inside the microfluidic chamber in function of the thickness of the layer (Fig. 2C.ii). The results from the numerical simulation suggests that using a 0.1 mm top sealing layer allows an oxygen level at 24h comparable to a PDMS device. Alternatively,

continuous perfusion of media may be used. Further studies taking oxygen permeability, and design variable into account will be undertaken.

Absorption of small molecules on PLA substrates

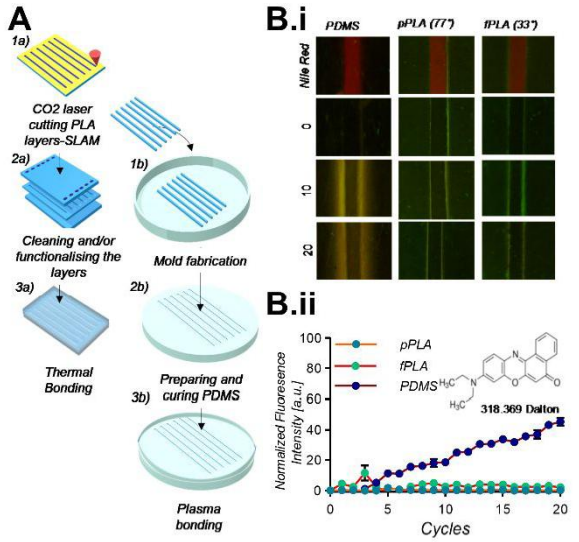
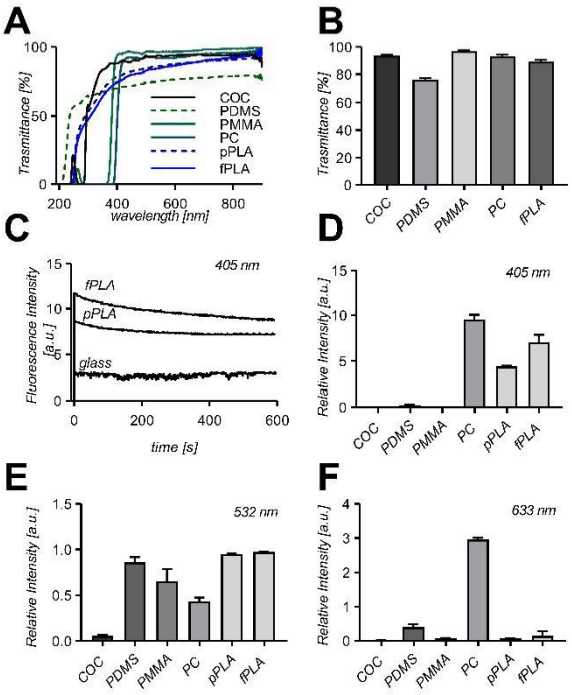


Figure 3 Investigation into the absorption of small molecules in PLA substrates. A) PLA and PDMS prototyping protocol. 1a) SLAM method is used to cut a channel of 1 mm width and access holes from a 1mm PLA sheet for channel and top layers, respectively. 2a) Top, bottom and channel layers are cleaned and functionalised.3a) The layers are bonded together 1b) The cut PLA parallelepipeds from 1a) are used as negative mould to fabricate the PDMS device to have the same channel cross-section for the different devices. 2b) The PDMS is mixed and placed in the mould and cured. 3b) Plasma bonding of the PDMS device. B) Absorption of hydrophobic compounds. B.i) top view of the channel for (from left to right) PDMS, pristine PLA and functionalized PLA. From the top to the bottom picture of the channel filled with a solution of Nile Red in ethanol (1µM), representing one of the reference point; then picture of the empty channel, representing the 0 reference point, and picture of the channel at the 10th and 20th cycle. B.ii) Normalized fluorescence intensity, measured at the centre of the channel, plotted in function of the cycle number. In insert molecular structure and molecular weight of Nile Red.

Several reports have shown that, unless treated, PDMS can absorb small hydrophobic compounds such as steroids and hormones [25–29]. This behaviour can be explained by the porous and hydrophobic nature of its polymeric network. This phenomenon can be a major drawback in bioassays, in particular drug testing assays where drugs engineered for rapid delivery are typically smaller than 500 Da and diffuse uncontrollably inside the microfluidic device [26,27]. Several methodologies have been proposed to overcome this issue; however, they result in delays in prototyping or manufacturing time. On the other hand, PLA, as a thermoplastic material should not show any absorption behaviour. In this study, the absorption of both hydrophilic and hydrophobic compounds was assessed in laser-cut *p*PLA and *f*PLA and PDMS channels. To test the absorption of hydrophobic compounds, Nile Red, a small hydrophobic fluorophore (318.37 Da), was selected as a representative molecule, while fluorescein salt (376.27 Da) was selected to test the absorption of a typical hydrophilic compound. The devices have been designed and manufactured with a channel cross-section of 0.8 mm² (Fig. 3A). A fluorescence image of each empty channel was taken with a microscope in order to create the null absorption reference point (Fig. 3B.i). To create the maximum absorption reference point, the channel was filled with the fluorescent solution (Fig. 3Bi). The solution was incubated inside the channel for 60s and then aspirated out of the channel from the outlet. Each channel was then cleaned with deionised water if loaded with Nile Red or with ethanol if loaded with fluorescein salt solution. A picture was taken after the cleaning cycle and the fluorescence intensity measured with the imaging software ImageJ and normalised with respect to reference points. The rinsing-cleaning-imaging cycle was repeated 20 times (Fig. 3B) and the normalised fluorescence intensity was plotted against the number of cycles (Fig. 3B.ii). This experiment showed PLA does not absorb hydrophobic or hydrophilic compounds, even when functionalised, while PDMS predictably absorbs small hydrophobic compounds (reaching 50% of absorption after 20 cycles), making PLA a superior material in this respect. In a preliminary study (data not shown), a hydrophilic, but cationic dye, Rhodamine 6G showed strong absorption in PDMS, and little or no absorption in PLA.



Optical properties of PLA: Transparency and autofluorescence

Figure 4. Investigation into PLA optical properties: A) UV-VIS spectrum of PLA (functionalised: fPLA and pristine PLA: pPLA) and different COC, PDMS, PMMA and PC materials; B) Comparison of light transmittance in the visible region of fPLA; compared to COC, PDMS, PMMA and PC substrate materials. C) Fluorescence decay profile under 600s continuum illumination of functionalised PLA, pristine PLA and glass; Autofluorescence intensity relative to glass of the different substrate materials after 60 s continuum illumination with 405 nm excitation D), 532 nm 633 nm excitation F).

In order to study the organ functionality and biological responses at the cellular and molecular level, the optical properties of an organ-on-chip system substrate material, are of fundamental importance and relevance [12]. It is well established that PDMS, PMMA, PC, COP and COC have good or excellent optical properties and transparency. Furthermore, they have a low fluorescence background or autofluorescence, when irradiated near UV wavelength, a desirable feature for fluorescent imaging [30]. The optical properties of PLA have been investigated and reported previously [31], however, very little is known about PLA autofluorescence properties. Here the transparency and autofluorescence of PLA substrate was investigated, analysing possible changes due to functionalisation (*p*PLA versus *f*PLA) in comparison with PDMS, PMMA, PC and COC substrates. We observed no difference between *p*PLA and *f*PLA with regards to transparency as transmittance of the light in the visible region (Fig. 4A). *f*PLA showed 92% of transparency, higher than PDMS, but lower with respect to the other materials tested. While PMMA showed the highest transparency (96 %), followed by COC, PC, PLA and PDMS (Fig. 4 B). In OOC applications fluorescence imaging is often required over a period of time rather than instantly [32]. Hence, the analysis of the autofluorescence over time under illumination is highly beneficial. Therefore, next, the autofluorescence was measured over a period of 600s (Fig. 4C). *p*PLA and *f*PLA were analysed and compared to glass, with data showing a gradual fluorescence decay for both. Contrary to the transparency behaviour, a notable difference was noted between the pristine and the functionalised PLA, with *f*PLA showing a higher autofluorescence due to the increased presence of –OH groups in the functionalised sample. The use of material without defect such as bubbles or lamellae is of importance to the reduction of the background autofluorescence. Furthermore, the autofluorescence of *p*PLA and *f*PLA were measured and compared to COC, PC, PDMS and PMMA with reference to glass. In these experiments, different wavelengths were tested, covering the range used in fluorescence microscopy. The fluorescence background was taken after 60s illumination[30]. As expected, for all the materials tested, the autofluorescence decreased with increasing wavelengths. An exception to this was PC, which demonstrated a lower fluorescence background at 532 nm. PLA showed autofluorescence levels no higher than ~ 1 times glass levels and comparable to the other test-ed materials (Fig. 4 D,E,F). Although the numerical fluorescence background measured for both pristine and functionalised PLA were higher than other materials, as shown in the biocompatibility section (Fig. 2), these results are not hindering PLA suitability for fluorescence imaging.

Cell tracking in a PLA Organ-On-Chip device Organ-On-Chip devices can be engineered in order to guide and spatially confine the cells. Recently, Biselli et al.[12] used an OOC device as a tool to study and quantitatively analyse the cancer-immune cells interactions via imaging analysis. The velocities, turning angle and path of the moving cells were used as indicator to study anticancer immune responses. Having demonstrated that the transparency of *f*PLA substrates is comparable

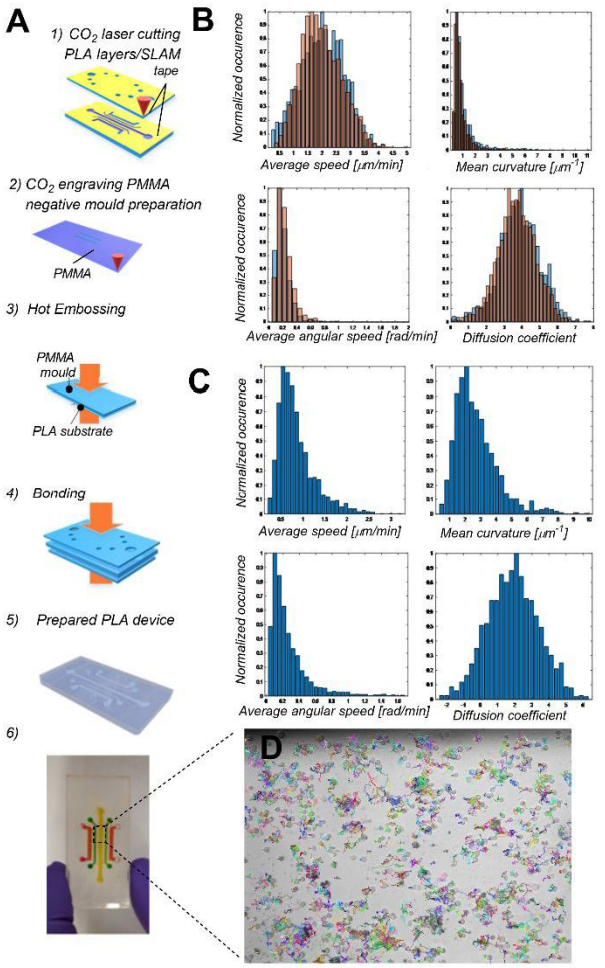


Figure 5 Demonstration of PLA suitability for cell tracking in Organ-on-a-chip and microfluidic cell culture devices. A) Schematization of the protocol to manufacture PLA Organ-on-a-chip or microfluidic devices. A-1) SLAM approach to create inlet and outlet holes in the top layer and to microstructure the channel on the middle layer. A-2) Rastering a PMMA substrate to create a mould for the bottom layer with diffusion channels. A-3) hot embossing the structure from the PMMA mould to PLA, using a temperature of 70 °C and a pressure of 0.5 MPa for 15 minutes. A-4) NaOH functionali-zation and UV activation of the surfaces with thermal bonding of the different layers. T=50 °C for 5 minutes. A-5) The assembled device is cooled down while maintaining the layers in contact. A-6) Photogragh of the final device filled with red, green and yellow food dyes B) Statistics of the kinematic descriptors for PC-3 prostate cancer cells on a PLA open disk (blue bars) and on a PS well plate (orange bars) C) Statistics of the Kinematic descriptors of the PC-3 prostate cancer cells on a PLA Organ-On-Chip device (image taken from the channel filled with yellow food dye). D) Extracted PC-3 prostate cancer cells trajectories through the Cell-hunter software on a PLA device.

to that of other common polymers used in microfluidic, we designed and manufactured an Organ-On-Chip device for cell

tracking in a confined channel. A device composed of three layers was manufactured using a combination of hot embossing and Sacrificial Layer laser Assisted Method (SLAM) approaches from *f*PLA substrates [7] (Fig. 5 A). In this experiment, prostate adenocarcinoma PC-3 cells were cultured on the device and a time lapse video was recorded as per material and method session in a CO_2 incubator at 37˚C. A microphotograph of the region of interest was taken at 1 minute intervals using a 4x magnification objective and videos analysed with a custom written software as per material and method session. Kinematic descriptors such as speed, mean curvature, and angular speed were extracted and compared for a PS Petri dish, PLA disc or inside a device. The kinematic descriptors between the PLA and the PS substrates were very similar despite slight difference of surface topology [33]. Additionally, the diffusion coefficient of individual cells was comparable on both substrates, thus demonstrating that cells on PS and PLA substrates have the same behaviour (Fig. 5B). On the other hand, the kinematic descriptors extracted from cell tracking in the PLA OOC device showed cell motility differences compared to the previous bare and open substrates (Fig. 5C). Cell random walk was observed, but as expected, subdiffusivity was noted in the confined environment. This might be made worse due to the difference in the oxygen permeability of the material. The differences observed in cell motility could be overcome by providing a better surface topology, a protein coating in culture channels to improve cell adhesion, and reducing the thickness of the device or applying media flow for gas exchange [34]. In conclusion, the transparency of *f*PLA substrates allowed advanced cell tracking in the device, similarly to the control PS Petri dish, and we were able to observe a random cell confined walk in PLA OOC device (Fig. 5D).

DISCUSSION

We performed a study to assess and fully characterize PLA suitability as a substrate material for microfluidic cell culture and Organ-On-Chip devices. We showed that PLA can offer many distinct advantages as a substrate for Organ-On-Chip applications over other polymers. Firstly, we showed that PLA could be functionalised via a rapid (60s) functionalisation process using NaOH, reducing its native contact angle of about 70 to a contact angle of 40°, suitable for cell culture. We found this surface treatment to be stable over at least 9 months, which shows commercial PLA devices treated in such a way would have excellent shelf-life. Secondly, we showed that cells could be cultured on PLA surfaces, as easily as on conventional polystyrene (PS) petri-dishes. Pristine and functionalised PLA performed better with regards to cell proliferation and cell toxicity compared to the majority of uncoated polymeric material such as PMMA, PC, COC. Thirdly, we demonstrated that PLA performed significantly better than PDMS with regards to the absorption of small molecules. Taking a hydrophilic representative molecule, Nile Red, of same size as oestrogen, a classic target compound in cell studies, we showed that PDMS absorbed 50% of the Nile Red after 20 load and wash cycles, while a similar PLA channel showed no absorption, whether or not it had been functionalised. Fourthly, we showed that lab-made PLA substrates are highly transparent and present very little auto-fluorescence, enabling high quality imaging, which is demonstrated through a cell-tracking experiment in an Organ-On-Chip device. Finally, advanced biological protocols such as random walk cell tracking were demonstrated in a PLA device. PLA is compatible with a range of complex integration, such as conducting electrodes, which we demonstrated in a previous publication [7]. While here we used a layer-by-layer approach to prototype Organ-On-Chip devices, PLA can be also formed, moulded, and printed. PLA is a popular material for filament deposition modelling, or 3D-printing and 3D printed microfluidic devices have demonstrated elsewhere [35]. However, these PLA 3D-printed devices currently lack the transparency necessary to enable high-resolution imaging and additional features are required to circumvent the lack of transparency. Here we showed that high PLA transparency can be achieved using moulding and laser-cutting techniques, which applies to both prototyping and high-volume manufacturing.

Up to now, other than ease of prototyping, one of the main reason for choosing PDMS for OOC application was its good oxygen permeability, with a diffusion coefficient of $3.5*10^{-9}$ m^2/s. In our cell culture experiments, we found the PLA oxygen diffusion coefficient of $2.5*10^{-12}$ m^2/s to be a limiting factor. However, continuous media perfusion or thinner lid are expected to remove this limitation as shown by our simulation results. Additionally, PLA lower oxygen diffusion coefficient enables the reduction and better control of water evaporation, and limit issues arising from osmolality differences induced by PDMS high oxygen diffusion coefficient.

The US Small Business Innovation Research, SBIR, has provided specific requirements for the assessment of novel material as replacement of PDMS. With respect to these requirements, PLA fulfills 12 (eight demonstrated in this paper) out of 16 individual criteria (Table S2 for further discussion). This confirms PLA suitability as next generation substrate material for advanced microfluidic cell culture and OOC applications.

CONCLUSION

In summary, we have demonstrated the suitability of PLA as a new substrate material for Organ-On-Chip devices. Our approach is designed to address many disadvantages inherent to conventional PDMS microfabrication technique. In conjunction with its biocompatibility, inertness to small molecules, optical qualities, PLA microfluidics will open opportunities to develop more sustainable solutions for Organ-On-Chip and microfluidic laboratories, in research or in industry. PLA represents a high-performing and more environmentally sustainable solution than other thermoplastic fossil-based materials proposed in the market. Ultimately, we envision that microfluidic device engineers will embrace a Design for Sustainability approach, and look into biopolymers, such as PLA, to develop sustainable single-use

devices, for a wide range of applications, from Organ-On-Chip to Point-Of-Care devices.

ASSOCIATED CONTENT

Supporting Information This material is available free of charge via the Internet at http://pubs.acs.org." Additional details on materials employed in OOC companies; AFM images of pristine and functionalized PLA; Details of biocompatibility experiments and cell death and proliferation on the different substrate employed in the study; Production of microchannels with sub-100 μm width; details of small molecules absorption experiment; details of autofluorescence experiments and autofluorescence noise created by defects in the materials; details of cell tracking experiments; Comparison of PLA properties with SBIR criteria.

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Author Contributions

AO, NH, VLC, VP and MKK, designed the research; AO, AMC, DV, AK, DDG, AM, LG, KW, VM, VP performed research; VS and DH contributed to reagents and analytic tools; AO, AK, DDG, NH, EM, VP, MKK analysed data; and AO and MKK wrote the paper. All authors read and reviewed the paper. All authors have given approval to the final version of the manuscript.

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SYNOPSIS TOC