Assessment of the biocompatibility of three-dimensional-printed polymers using multispecies toxicity tests

Feng Zhu,¹ Timo Friedrich,² Dayanthi Nugegoda,^{3,4} Jan Kaslin,² and Donald Wlodkowic^{1,4,5,a)}

¹*The BioMEMS Research Group, School of Applied Sciences, RMIT University, Melbourne, Victoria 3083, Australia*

²*Australian Regenerative Medicine Institute, Monash University, Clayton, Victoria 3800, Australia*

³*Ecotoxicology Research Group, School of Applied Sciences, RMIT University, Melbourne 3083, Australia*

⁴*Centre for Environmental Sustainability and Remediation (EnSuRe), RMIT University, Melbourne, Victoria 3083, Australia*

⁵*Centre for Additive Manufacturing, RMIT University, Melbourne, Victoria 3083, Australia*

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Additive manufacturing was adopted in multiple fields of life sciences. It is also becoming a popular tool for rapid prototyping of microfluidic and biomedical devices. Limited studies have been performed to investigate the biological implications of using 3D printed polymers. Here we assessed the biocompatibility of seven commercially available polymers, using a battery of standardized bioassays for chemical risk assessment. Our data show that leachates from photopolymers substrata appear to be very toxic to vertebrates and several invertebrate indicator organisms. These results demonstrate significant consequences for the use of selected photopolymers in the fabrication of bio-devices. © 2015 AIP Publishing LLC.
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INTRODUCTION

Additive manufacturing technologies are becoming widely available and allow rapid prototyping and fabrication of the next generation of microfluidic chip-based technologies.^{1–5} The capability to perform rapid design troubleshooting and fabricate monolithic devices without any need of assembly processes is a convincing reason towards a wider adoption of additive manufacturing in the Lab-on-a-Chip field.^{6–11} In this regard, biocompatibility of currently available 3D printed polymers is critical for any biological and biomedical applications.^{10,11} Bioassays on living cells and tissues are conducted in aqueous media. Photopolymers such as acrylates and epoxy resins commonly used in 3D printing exhibit, however, swelling in contact with water-based media.^{10,12} Moreover, only limited research has been completed on the toxicity of 3D materials to cells, tissues, and model organisms.^{5,11,12}

We present a comprehensive toxicity profiling of a panel of common polymers used in 3D printing applications. For profiling we chose five standard whole-organism biotests spanning diverse evolutionary and trophic levels such as algae, zooplankton, fish embryos, and larvae. The main motivation of our work was to evaluate toxicity using standardized aquatic biotests that eliminate issues related to adsorption of cell culture media to polymer surfaces. Our work for the first time provides an in-depth and multispecies view of potential biological implications of fabricating devices using additive manufacturing technologies. The purpose of this work is

^{a)} Author to whom correspondence should be addressed. Electronic mail: donald.wlodkowic@rmit.edu.au

not only to guide scientists as to the biocompatibility of specific polymers used in the field of additive manufacturing but also to demonstrate rapid methods to assess their toxicity.

MATERIALS AND METHODS

Additive manufacturing

3D structures were designed and modeled using SolidWorks 2011 (Dassault Systems SolidWorks Corp, USA) CAD package.¹¹ Each structure had a surface area of 6250 mm². A fused deposition modelling (FDM) process was used with a Replicator 2 (MakerBot, USA) printer in poly(lactic) acid (PLA) and uPrint SE (Stratasys, USA) printer in acrylonitrile butadiene styrene (ABS). Multi-Jet Modelling (MJM) process was used with a HD3500 Plus (3D Systems, USA) in a VisiJet Crystal resin (3D Systems).¹¹ Stereolithography (SLA) processes were used with: (i) Viper Pro (3D Systems) using Watershed 11122XC (DSM Somos, USA) or Dreve Fototec 7150 Clear (Dreve Otoplastik) resins; (ii) ProJet 7000HD using VisiJet SL Clear resin (3D Systems); and (ii) Form 1 using Clear resin (FormLabs, USA) as described earlier.¹¹

Leachate extraction protocol

3D printed structures with a total surface area of 25 000 mm² were placed in a glass flask filled with 500 ml of aqueous solution of respective culture medium. Surface to volume ratio mirrored that found in a standard 24-well microtiter culture plate. The leachate extraction was performed in darkness at 22 °C for up to 72 h. All leachates were immediately used in selected biotests.

Toxicological profiling

Five commonly used indicator organisms were selected for testing of potential chemical hazard risks associated with 3D printed polymer leachates: (i) OECD 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test (Algaltoxkit-F Selenastrum; MicroBioTests, Inc., Belgium),^{13,14} (ii) ASTM E1440–91 Acute Toxicity Test with the Rotifer *Brachionus* (Acute Rotoxkit-F; MicroBioTests, Inc.),^{14–16} (iii) OECD 202 *Daphnia* sp. Acute Immobilisation Test (Daphtoxkit-F; MicroBioTests, Inc.),^{13,14,17} (iv) USEPA *Ceriodaphnia* sp. Acute Toxicity Test (Acute Ceriodaphtoxkit-F; MicroBioTests, Inc.),¹⁴ (v) OECD 236 Fish Embryo Acute Toxicity (FET) Assay with *Danio rerio*,^{13,18,19} (v) fish behavioral toxicity test using larval zebrafish (*Danio rerio*) stages at 7 days post fertilisation (dpf). The ZebraBox system (ViewPoint Inc) was used for tracking individual larval swimming distance in 5 min intervals. A total of 10 intervals for 20 individuals were graphed using Excel.

RESULTS AND DISCUSSION

All polymer leachates caused a significant growth inhibition in the freshwater micro-algae *Pseudokirchneriella subcapitata* and the effects indicated an exposure-dependent relationship with a limited response during the first 24 h (Fig. 1(a)). Poly(lactic) acid and acrylonitrile butadiene styrene despite their perceived safety induced nearly 50% growth inhibition after 96 h of exposure. SLA polymers such as Watershed 11122XC, Dreve Fototec 7150 Clear, and VisiJet SL Clear induced greater than 90% of growth inhibition (Fig. 1(a)). VisiJet Crystal and Form 1 Clear polymers induced approximately 70% and 60% of growth inhibition, respectively. Acute toxicity tests with the rotifer *Brachionus calyciflorus* revealed 100% mortality in response to all leachates apart from PLA, ABS, and VisiJet Crystal. The latter were not toxic to rotifers over the standard exposure time of 24 h (Fig. 1(b)). We also explored the toxicity of polymer extracts using the freshwater crustacean *Daphnia magna*, one of the most sensitive and frequently used aquatic toxicity bioassays. The experimental results revealed that extracts from SLA polymers such as Watershed 11122XC, Dreve Fototec 7150 Clear, and VisiJet SL Clear resins were toxic to *Daphnia* sp. neonates. All of them induced 100% mortality after 24 h of exposure to aqueous extracts (Fig. 1(c)). Interestingly, both VisiJet Crystal and Form 1 Clear extracts did not induce any statistically significant mortality after 24 h of exposure. Their effects appeared to be delayed because by 48 h, the survival rate was ca. 50%, and all specimens were dead after 72 h (Fig. 1(c)). This heterogeneity in responses combined with results

FIG. 1. Toxicity profiling of 3D printed polymer extracts using invertebrate models and performed according to OECD Guidelines for Testing of Chemicals. (a) OECD 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test on freshwater micro-algae *Pseudokirchneriella subcapitata*; (b) ASTM E1440-91 Acute 24-h Toxicity Test with the rotifer *Brachionus calyciflorus*; (c) OECD 202 *Daphnia magna* Acute Immobilisation Test; (d) USEPA *Ceriodaphnia dubia* Acute 24-h Toxicity Test. ABS—acrylonitrile butadiene styrene; PLA—poly(lactic) acid; VJ—VisiJet Crystal; WS—Watershed 11122XC; DV—Dreve Fototec 7150 Clear; VC—VisiJet SL Clear; F1—Form 1 Clear. Results represent data from at least three independent experiments performed in triplicate.

from algae growth inhibition assays suggests species-dependent responses to water-soluble leachates of certain polymers used in additive manufacturing. Acute toxicity tests over 24 h with the freshwater crustacean *Ceriodaphnia dubia* provided statistically similar results to those obtained on rotifers (Fig. 1(d)). Tests performed using a standard OECD FET test on developing zebrafish embryos revealed significant toxicity with all SLA and MJM polymers. By 24 h, the embryo survival rate was ca. 10% for VisiJet Crystal and 0% for the remaining SLA polymers (Fig. 2(a)). However, it was observed that 100% of zebrafish embryos exposed to ABS and PLA extracts survived and developed normally for up to 72 h (Fig. 2(a)). Mortality of 5 days old zebrafish larvae grown in polymer extracts was statistically similar to FET test results (Fig. 2(b)). High toxicity of SLA polymers detected using conventional mortality-based assays on invertebrate and vertebrate models prompted us to explore very short duration exposures using dynamic behavioral toxicity parameters. The latter are increasingly postulated as alternatives to traditional mortality testing. Behavioral perturbations can precede mortality endpoints at considerably lower concentrations and are therefore postulated as new indicators of sub-lethal toxic effects of chemicals to biota. Interestingly, behavioural toxicity analysis revealed a sudden and complete paralysis of zebrafish larvae just 5 min after exposure to all photopolymers used in SLA and MJM processes (Figs. 2(c) and 2(d)). Occurrence of such dramatic hypoactivity syndrome indicates that photopolymer leachates directly affect the central nervous system or muscle contraction in zebrafish larvae. Leachates from ABS and PLA did not induce any behavioural abnormalities (Figs. 2(c) and 2(d)). This behavioural bioassay enabled us to determine which leachates from 3D printed structures have instantaneous sub-lethal toxicity.

CONCLUSIONS

In summary, we used well-established and very sensitive aquatic bioindicators and deployed them as proxies to evaluate toxicity of polymers used in the 3D printing processes. Based on our results, we conclude that many of the polymerized resins used in MJM and SLA processes are unsafe, as they appear to leach toxic substances to water-based culture media. This is surprising as, for example, VisiJet Crystal was recently classified as a substance with

FIG. 2. Toxicity profiling of 3D printed polymer extracts using vertebrate zebrafish model according to OECD Guidelines for Testing of Chemicals. (a) OECD 236 Fish Embryo Acute Toxicity (FET) Assay with *Danio rerio*. (b) Mortality tests on 7 dpf zebrafish larvae exposed to polymer extracts. Data were obtained using manual scoring of heartbeat presence. (c) Zebrafish larvae trajectories after 5 min exposure to polymer extracts. (d) Behavioural responses of zebrafish larvae represented as the change in total distance traveled ($\pm$ standard error), exposed for 5 min to selected polymer extracts. ABS—acrylonitrile butadiene styrene; PLA—poly(lactic) acid; VJ—VisiJet Crystal; WS—Watershed 11122XC; DV—Drewe Fototec 7150 Clear; VC—VisiJet SL Clear; F1—Form 1 Clear. Results represent data from at least three independent experiments performed in triplicate.

favorable biocompatibility as evidenced by United States Pharmacopeia (USP) Class VI certification. While advances in additive manufacturing of microfluidic and biomedical devices enable a rapidly growing number of applications, our results suggest that considerable caution has to be exercised due to potential biocompatibility problems.

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