A customizable 3D printed device for enzymatic removal of drugs in water

Xiaoyan Xu[1,a], Tomás Pose-Boirazian[2,a], Gemma Eibes[3], Laura E. McCoubrey[1], Jose Martínez-Costas[2], Simon Gaisford[1,4], Alvaro Goyanes[4,5,*], Abdul W. Basit[1,4,*]

[1] Department of Pharmaceutics, UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1N 1AX, UK.

[2] Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CiQUS), Departamento de Bioquímica y Biología Molecular, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain.

[3] CRETUS Institute, Dept. of Chemical Engineering, Universidade de Santiago de Compostela,

15782 Santiago de Compostela, Spain.

[4] FabRx Ltd., 3 Romney Road, Ashford, Kent TN24 0RW, UK.

[5] Departamento de Farmacología, Farmacia y Tecnología Farmacéutica, I+D Farma (GI-1645), Facultad de Farmacia, and Health Research Institute of Santiago de Compostela (IDIS), Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain.

[a] Both authors contributed equally to this work

* Correspondence: a.basit@ucl.ac.uk ; a.goyanes@fabrx.co.uk

Abstract

The infiltration of drugs into water is a key global issue, with pharmaceuticals being detected in all nearly aqueous systems at often alarming concentrations. Pharmaceutical contamination of environmental water supplies has been shown to negatively impact ecological equilibrium and pose a risk to human health. In this study, we design and develop a novel system for the removal of drugs from water, termed as Printzyme. The device, fabricated with stereolithography (SLA) 3D printing, immobilises laccase sourced from *Trametes Versicolor* within a poly(ethylene glycol) diacrylate hydrogel. We show that SLA printing is a sustainable method for enzyme entrapment under mild conditions, and measure the stability of the system when exposed to extremes of pH and temperature in comparison to free laccase. When tested for its drug removal capacity, the 3D printed device substantially degraded two dissolved drugs on the European water pollution watch list. When configured in the shape of a torus, the device effectively removed 95 % of diclofenac and ethinylestradiol from aqueous solution within 24 and 2 hours, respectively, more efficiently than free enzyme. Being customizable and reusable, these 3D printed devices could help to efficiently tackle the world's water pollution crisis, in a flexible, easily scalable, and cost-efficient manner.

Keywords

Three-dimensional printing; Stereolithographic fabrication; Additive manufacturing; Enzyme immobilization; Pharmaceutical remediation; Bioremediation

1. Introduction

It is now common, and almost expected, that an individual will take dozens of medicines in their lifetime, especially with advancing age (Age UK, 2019; Mieiro et al., 2019; National Center for Health Statistics, 2019). As more medicines are consumed, more are consequentially excreted or disposed of, often permeating the world's water. Drugs and their metabolites are present in nearly all aquatic environments, from drinking water, to ground water, sea water, and waste water with concentrations generally in the range of ng to μg/L (Patel et al., 2019). However, at certain sites like hospital effluents or production facilities, concentrations can reach the high mg/L range (Larsson et al., 2007; Wiest et al., 2018). The pollution issue is global, with countries recording significant concentrations of pharmaceuticals in water across Europe, Africa, Asia, and the Americas. Presently, antibiotics, analgesics, lipid-lowering drugs, and oestrogens are the most reported water-polluting drugs worldwide; however the true number and scale of drugs is likely larger than currently known, due to limitations and priorities in testing (aus der Beek et al., 2016). This insidious infiltration of pollutants poses a significant risk to global health and ecological equilibrium. For example, diclofenac is known to be one of the most prolific drugs entering water, with a global average of 0.032 μg/L present in all surface water compartments (aus der Beek et al., 2016). The entrance of diclofenac into wild food chains is lethal for migratory birds, such as vultures. In fact, diclofenac has resulted in the population collapse of three vulture species in South Asia and poses a significant threat to several European species (Margalida and Oliva-Vidal, 2017; Swan et al., 2006). Elsewhere in the environment, the occurrence of oestrogens in sewage effluents has led to feminisation of male fish, dramatically impacting reproductive success (Auriol et al., 2006; Gross-Sorokin et al., 2006; Mills et al., 2015; Pérez-Coyotl et al., 2019). With regards to human health, the practice of irrigating crops with wastewater increases risk of consumer genotoxicity, due to the presence of solubilised antineoplastics (Russo et al., 2019). Moreover, antibiotics in water are thought to increase rates of bacterial antibiotic resistance, a currently

critical challenge that threatens global health as whole (Mishra et al., 2018; Singh et al., 2020; World Health Organization, 2020). Even drugs of abuse are infiltrating water, as highlighted by studies in Madrid and Changzhou City, East China, which found cocaine, opioids, and methamphetamine in the cities' drinking supply and wastewater (Deng et al., 2020; Mendoza et al., 2016).

In the face of this challenge, the World Health Organization (WHO) has launched a working group to address how pharmaceutical burden in water can be lessened. As such, several methods for removing drugs from water have been proposed, including sludge processes; biofiltration; reverse osmosis; ozonation; chlorination; and nanofiltration. Unfortunately, many of these prosses are variably efficient – with drug removal rates sometimes lower than 10% - and often require expertise with access to expensive and sizeable equipment (World Health Organization, 2011). Thus, there is a global requirement for efficacious technologies that can remove drugs in water simply and cost-effectively. Ideally, methods should also work reliably in a range of different environments, regardless of pH, temperature, or physical space. As such, technologies that can be customised to fit into different physical spaces (such as pipes or machinery) are desirable.

3D printing is a key manufacturing method for customisable devices (Awad et al., 2020; Elbadawi et al., 2020; Vivero-Lopez et al., 2021; Xu et al., 2021c; Xu et al., 2020). Among different categories of 3D printing technologies, vat photopolymerisation-based 3D printing is a type of technique that creates physical objects from a vat of liquid resins under light irradiation in a layer-by-layer fashion, offering superior resolution and a smooth surface finish that other 3D printing technologies cannot compete with (Ng et al., 2020; Xu et al., 2021a; Xu et al., 2021b). In this study, we develop, manufacture, and validate a 3D printed biocatalytic device (termed Printzyme) for the removal of drugs from wastewater at concentrated point sources such as hospitals or pharmaceuticals facilities. The Printzyme has fully modifiable morphology, and utilises the natural enzyme laccase, sourced from *Trametes Versicolor*, as a drug oxidating agent (Yang et al., 2017).

Laccases are ubiquitously found in nature, and have been shown to effectively degrade a wide variety of pharmaceuticals, including several classes of antibiotics, paracetamol, diclofenac, naproxen, anti-depressants, and oestrogens (Ba et al., 2014; Lloret et al., 2010; Marco-Urrea et al., 2010) (Tahmasbi et al., 2016) (Auriol et al., 2008; Becker et al., 2017; Tamagawa et al., 2006). Printzyme entraps laccase in a hydrogel via stereolithography (SLA) 3D printing. The novel process immobilises the enzyme and simultaneously prints under mild conditions, representing an inexpensive and sustainable manufacturing process. We highlight Printzyme's optimal conditions of use, stability at varying pH and temperature readings, and homogenous laccase immobilisation. Crucially, we demonstrate Printzyme's ability to remove diclofenac sodium and an oestrogen, ethinylestradiol, from water with excellent device reusability.

2. Materials and methods

2.1 Materials

Laccase from *Trametes Versicolor* (activity 0.5 U/mg when using catechol as substrate at pH 6, equivalent to 1.65 U/mg using ABTS at pH 3), poly(ethylene glycol) diacrylate (PEGDA, average Mn 575), diclofenac sodium (Mw 318.13 g/mol), ethinylestradiol (Mw 296.40 g/mol) and the photoinitiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Mw 294.21 g/mol) were purchased from Sigma-Aldrich (Dorset, UK). 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Citric acid monohydrate (Mw 210.15 g/mol) and di-sodium hydrogen orthophosphate dodecahydrate (Mw 358.137 g/mol) were acquired from Fisher Scientific (Loughborough, UK). All reagents were used as received.

2.2 3D design

Printzymes were designed digitally in four shapes: cylinders (6 mm diameter x 3-4 mm thickness) and toruses (6 mm outer diameter x 5 mm inner diameter x 3-4 mm thickness) using 123D Design

software (Autodesk Inc., USA). Thickness was varied to allow assessment of how surface area affected enzyme activity in the system. To allow designs to be read by the SLA printer, they were exported as stl. files.

2.3 Formulation and 3D printing of Printzyme

Photopolymer resins for SLA printing were composed of laccase, a photoinitiator (LAP), and a mixture of PEGDA and water at different mass ratios (Table 1). Laccase was added to the initial PEGDA/water mixture together with 0.5% w/w of LAP, followed by stirring at room temperature until complete dissolution. This enzyme-loaded resin was subsequently poured into a resin tank for printing. Using a commercial Form 2 SLA 3D printer (Formlabs Inc., USA) equipped with a 405 nm laser, all Printzymes were directly printed onto the printing platform. Printer settings were 'Clear 04' for material and 0.1 mm for layer thickness. The total printing time was 1 hour 30 minutes and more than 50 Printzymes can be fabricated at the same time. After printing, Printzymes were washed with isopropyl alcohol for 2 minutes in a Form Wash (Formlabs Inc., USA), followed by post cure in a Form Cure (Formlabs Inc., USA) for 20 min. This step was to remove the presence of any unreacted PEGDA monomers, LAP, or free laccase, on the device surface. All Printzymes were stored at 4 °C until further use.

Table 1. Composition of the photopolymer resin

Printzyme	Laccase from *T. versicolor* (% w/w)	PEGDA:Water ratio	PEGDA (% w/w)	Water (% w/w)
Printzyme 0.4-20	0.4	20:80	19.82	79.28
Printzyme 0.4-50	0.4	50:50	49.55	49.55
Printzyme 4-20	4.0	20:80	19.10	76.40

Printzyme 4-50	4.0	50:50	47.75	47.75

*each resin includes 0.5% w/w LAP

2.4 Environmental scanning electron microscopy (ESEM)

Printzymes were sliced in half and subsequently attached to a self-adhesive carbon disc mounted on a 25 mm aluminium stub with silver paint. An environmental scanning electron microscope (FEI Quanta 200 FEG, UK) in ESEM mode, at 200 Pa pressure, 20 kV accelerating voltage, and a large filled detector, was employed to obtain detailed images of the cross-section of the printed device.

2.5 Enzyme activity assay

The activities of free laccase and Printzyme were determined by a colorimetric assay. Here, 0.266 mM ABTS was used as a laccase substrate, dissolved in citric phosphate buffer composed of 0.1 M citric acid buffer and 0.2 M dibasic sodium phosphate buffer. When 3 mL of this reaction medium was incubated with either free laccase (0.4% w/v aqueous solution) or one Printzyme of known weight, the enzymatic oxidation of ABTS was followed by measuring the incubation medium's change in absorbance at 420 nm using a Jasco V-770 UV-visible/NIR spectrophotometer. Hence, the extent of ABTS oxidation was used as a measurement of laccase activity. Effective activity, in mU/mg, was that observed when using the Printzyme. All measurements were performed in triplicate. Immobilization yield was calculated as:

$$IY(\%) = \frac{A_P}{A_0} \cdot 100 \qquad (1)$$

where A_0 is the theoretical loaded activity, and A_P represents the total laccase activity on the Printzyme. To determine A_P, the Printzyme was shredded into fine particles and the activity of the particles was measured. Five replicates were use to obtain this activity.

The effectiveness factor (dimensionless) is the ratio between enzymatic activity monitored in immobilized state compared to free solution:

$$\eta = \frac{reaction\ rate\ Printzyme}{reaction\ rate\ free\ enzyme} \tag{2}$$

2.6 Optimal pH and temperature for free laccase and Printzyme activities

To determine optimal pH of enzyme activity, free laccase and one Printzyme were separately incubated in 3 mL citric phosphate buffer + 0.266 mM ABTS, for 5 minutes at 25 °C. Enzyme activity of both free laccase and Printzymes were assessed as in Section 2.5. The pH of the citric phosphate buffer was varied from pH 3.0 – pH 6.0. The maximal reading for ABTS oxidation was considered as 100% enzyme activity, and relative activity was calculated as the ratio between the activity at a measured pH and the maximum activity. All measurements were performed in triplicate.

The optimal temperature for enzymatic activity was determined by incubating both free laccase and one Printzyme at temperatures ranging from 30 - 80 °C, for 5 minutes in 3 mL citric phosphate buffer + 0.266 mM ABTS at pH 3.0. Enzyme activity was assessed as in Section 2.5. The maximal reading for ABTS oxidation was considered as 100% enzyme activity, and relative activity was calculated as the ratio between the activity at a measured temperature and the maximum activity. All measurements were performed in triplicate.

2.7 Effect of pH and temperature on free laccase and Printzyme stability

The 24-hour stability of both free laccase and Printzyme at selected pH readings (pH 3.0, pH 4.0, pH 6.0, pH 8.0) was determined with the enzyme activity assay outlined in Section 2.5. Free laccase and 60 mg Printzyme 0.4-20 were incubated in 3 mL citric phosphate buffer (or phosphate buffer for pH 8.0) + ABTS for 24 hours at room temperature. Samples of the incubation medium were taken at defined timepoints (1 hour, 3 hours, 6 hours, 24 hours), and their enzymatic activity

was measured immediately. The reading at 0 hour was considered as 100% enzyme activity, and relative activity was calculated as the ratio between the activity for a specific reading and the maximum activity. All measurements were performed in triplicate.

The 24-hour stability of both free laccase and Printzyme at defined temperatures (25 °C, 40 °C, 50 °C, 60 °C) was determined with the enzyme activity assay outlined in Section 2.5. Free laccase and 60 mg Printzyme 0.4-20 were incubated in 3 mL citric phosphate buffer (pH 6.0) + ABTS for 24 hours at room temperature. Samples of the incubation medium were taken at defined timepoints (1 hour, 3 hours, 6 hours, 24 hours), and their enzymatic activity was measured immediately. The reading at 0 hour was considered as 100 % enzyme activity, and relative activity was calculated as the ratio between the activity for a specific reading and the maximum activity. All measurements were performed in triplicate.

2.8 Reusability studies

Printzyme is intended as a reusable device. Thus, reusability was investigated by performing 18 isolated uses of one Printzyme lasting 5 minutes each. ABTS, the standard substrate for determination of laccase activity, was used as model substrate. After each cycle, the Printzyme was filtered and washed with distilled water and subsequently used in the next iteration. Enzymatic activity was assessed after each use using the method outlined in Section 2.5. The activity of the enzyme in the initial cycle was considered as 100% and the relative activity was calculated as the ratio between the activity at each cycle and the initial activity [67]. All measurements were performed in triplicate.

2.9 Removal of pharmaceuticals by Printzyme

The ability of Printzyme 4-20 to remove drugs from water was tested using two model drugs, diclofenac and ethinylestradiol. Separate solutions of the two drugs were prepared in phosphate buffer (0.1 M, pH 6.0) at concentrations of 2.5 mg/L. 350 mg Printzyme 4-20 was deposited into

each drug solution (50 mL) inside a 100 mL flask, and continuously shaken at 100 rpm for 48 hours. Experiments with free laccase were carried out usin equal activity (≈ 340 U/L) as a comparison. These reaction mixtures were maintained at room temperature for the duration of experimentation, and samples were withdrawn at specific timepoints (2 hours, 4 hours, 8 hours, 24 hours, 48 hours). The concentration of drug in each withdrawn sample was determined using high performance liquid chromatography (HPLC) (Section 2.10). Control studies were run in parallel, whereby Printzyme formulations without laccase were used in place of active Printzymes. Measurements were performed in duplicate. After the experiment, all the Printyzmes were recovered from the flasks by removing the drug solution and pictures were taken.

2.10 High-performance liquid chromatography (HPLC) analysis

A HPLC system (Hewlett Packard 1260 Series HPLC system, Agilent Technologies, Cheadle, UK) was used to measure the concentration of diclofenac or ethinylestradiol in samples exposed to Printzyme 4-20 for defined periods of time (Section 2.9). For both drugs, an Eclipse plus C18 column, 100 × 4.6 mm (Zorbax, Agilent technologies, Cheshire, UK) was used as the stationary phase. The mobile phase consisted of phosphate buffer (50 mM, pH 4.5) with acetonitrile at isocratic conditions of 50:50 for diclofenac, and 40:60 for ethinylestradiol. For both drugs, sample injection volume was 100 µL and mobile phase flow rate was 1.0 mL/min. Diclofenac retention time was 6 minutes and eluent absorbance was measured at 220 nm. For ethinylestradiol, drug retention time was 10 minutes, and an absorbance of 210 nm was used for detection.

2.11 Statistical analysis

Data from the removal of pharmaceuticals by Printzyme were statistically analysed by performing t-tests: two-sample assuming equal variances (Microsoft Excel, Microsoft, Redmond, WA, USA). $P < 0.05$ was considered statistically significant. Significance level notation was expressed as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p < 0.0001$, and NS for not significant.

3. Results and discussion

3.1 Formulation and 3D printing of Printzyme

Four formulations were initially investigated for the optimal Printzyme device (Table 1): Printzyme 0.4-20 (0.4 % laccase, 20:80 PEGDA:water); Printzyme 0.4-50 (0.4% laccase, 50:50 PEGDA: water); Printzyme 4-20 (4.0% laccase, 20:80 PEGDA:water); and Printzyme 4-50 (4.0% laccase, 50:50 PEGDA:water). PEGDA was selected as the formulation support due to its flexibility, biocompatibility, ease of crosslinking, and uniform pore size (Blanchette et al., 2016; Li et al., 2015; Steier et al., 2020). LAP was chosen as the SLA photoinitiator due to its high water solubility and cytocompatibility (Fairbanks et al., 2009).

During the formulation compounding stage, it was observed that the formulation intended as Printzyme 4-50 appeared as a suspension. This signifies that the laccase was not fully dissolving in the PEGDA-water solution, a trait that would likely cause heterogenous distribution of the enzyme in the printed device, and thus affect the reproducibility of enzyme activity in the final Printzyme. For this reason, the Printzyme 4-50 formulation was removed from the study. The remaining three formulations were observed to form solutions and were successfully printed into two morphologies using SLA 3D printing, as shown in Figure 1.

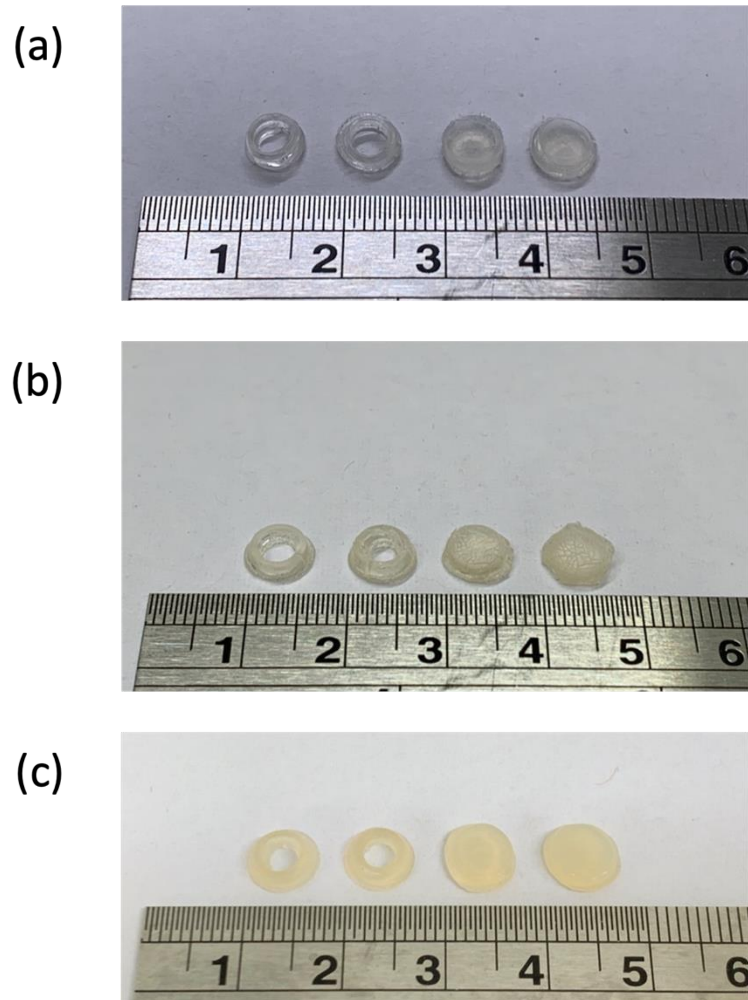


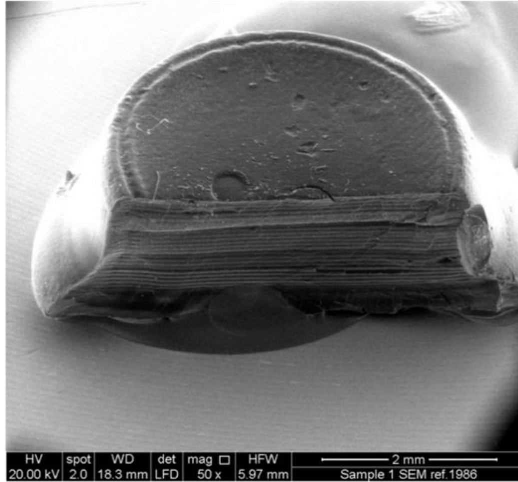
Figure 1. Photographs of two toruses and two cylinders fabricated from (a) Printzyme 0.4-20, (b) Printzyme 0.4-50, and (c) Printzyme 4-20, via SLA 3D printing. The featured scale is in cm.

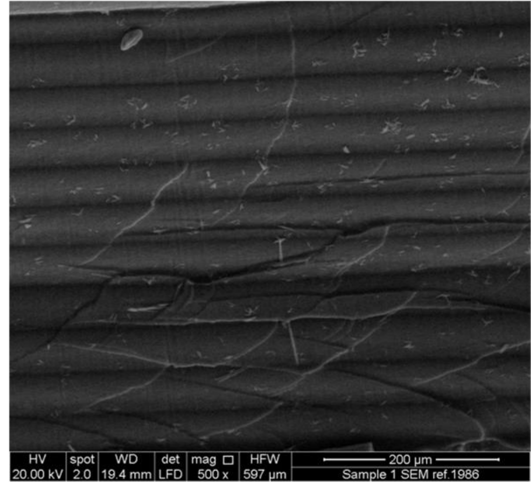
Two different shapes of Printzyme, a cylinder and torus, were designed and printed to evaluate the effect of surface area to volume ratio on immobilised laccase activity. As seen in Figure 1, Printzyme 4-20 has an opaque appearance corresponding to its higher laccase content (4.0% w/w) compared to the more translucent Printzyme 0.4-20 and 0.4-50 formulations (containing 0.4% w/w laccase). A PEGDA to water ratio of 20:80 was found to produce Printzymes with adequate resolution and consistency in shape. Correspondingly, the Printzyme 0.4-50 formulation, with its higher PEGDA content, resulted in decreased resolution. Whilst the thickness

of the designs was set to 3 or 4 mm, some shrinkage did occur after 3D printing, resulting in a loss of around 0.5 mm thickness.

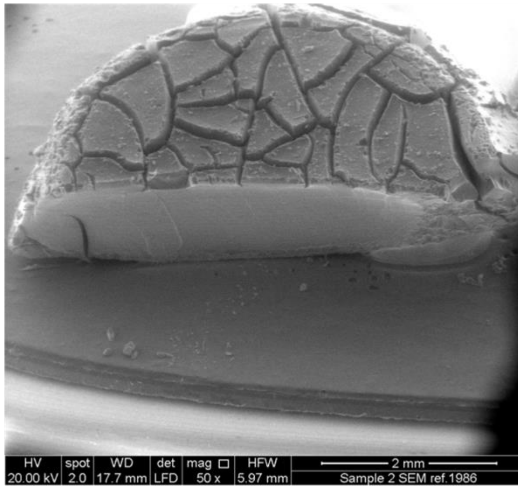
ESEM allowed detailed inspection of the Printzyme surfaces (Figure 2). From the ESEM images, it is clear that the resolutions of Printzymes 0.4-20 and 4-20 are superior to Printzyme 0.4-50 (Figure 2b). Printzyme 0.4-50 shows a fractured surface, most likely due to its lower water content. Previously, it has been reported that an increase in PEGDA concentration or decrease in water content in hydrogel formulations results in a reduction of the curing time required during SLA printing (HuangáGoh and HoseináSakhaei, 2018; Madzarevic et al., 2019). With this in mind, optimal SLA printing of Printzyme 0.4-50 likely requires a lower SLA curing time than Printzymes 0.4-20 and 4-20 (Figures 2a and 2c). For all Printzymes, ESEM shows the layers formed in the devices' internal structures, caused by the layer-by-layer curing method of the SLA printer (Figure 2).

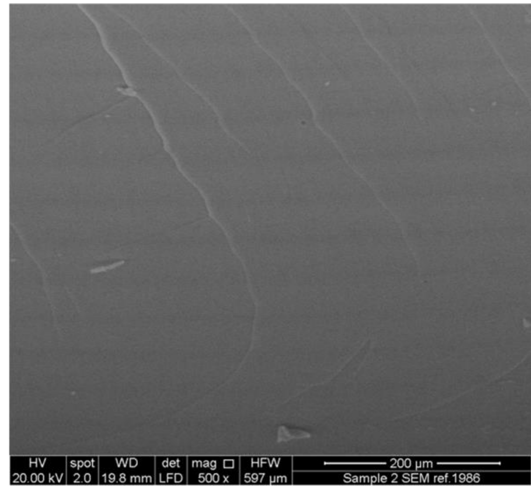
(a)



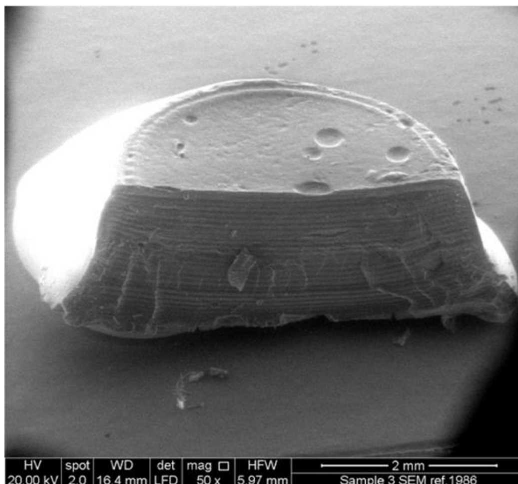


(b)





(c)



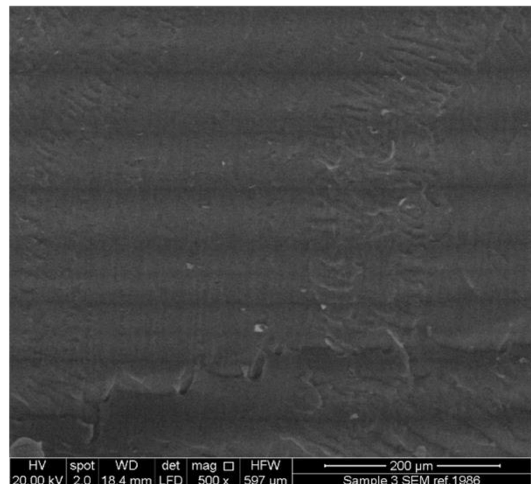


Figure 2. ESEM images of cylinder fabricated from (a) Printzyme 0.4-20, (b) Printzyme 0.4-50, and (c) Printzyme 4-20 via SLA 3D printing.

3.2 Initial enzymatic activity assessment of Printzymes

The poor resolution and surface fractures observed from the imaging of Printzyme 0.4-50 led to fluctuating laccase activity, most probably due to the structural cracks exposing variable amounts of enzyme active sites. As a result, Printzyme 0.4-50 was withdrawn from the study, as consistency of enzymatic activity is a key objective for the final device. In comparison, Printzyme 0.4-20 and 4-20 achieved reproducible levels of laccase activity (Table 2). The maximum specific activity of the devices was measured after shredding the Printzymes into fine particles, in order to facilitate display of enzyme active sites. Table 2 shows that the process of SLA printing had a minor effect on enzyme activity, since the Printzyme constructs retained more than 75% of the activity of laccase incorporated in the formulation prior to printing. The immobilisation yields (Eq. 1) attained here agree with previously reported works. For instance, laccase encapsulated in a silica sol-gel matrix demonstrated immobilisation yields of 83 to 59% (Lloret et al., 2011).

Table 2. Immobilization yield and effective activity of the different Printzyme configurations

	Printzyme 0.4-20		Printzyme 4-20	
Activity added[1] (mU/mg)	6.60 ± 0.23		66.0 ± 2.3	
Maximum activity[2] (mU/mg)	5.03 ± 0.54		49.60 ± 6.90	
Immobilisation yield[3] (%)	76.20 ± 10.83		75.20 ± 13.08	
	Cylinder	Torus	Cylinder	Torus

Apparent activity[4] (mU/mg)	4mm thickness	0.042 ± 0.013	0.109 ± 0.021	0.501 ± 0.027	1.022 ± 0.074
	3mm thickness	0.240 ± 0.020	0.344 ± 0.017	1.377 ± 0.132	1.426 ± 0.068

[1] Theoretical initial activity, based on mg of enzyme added (1.65 U/mg enzyme)

[2] Printzyme was shredded into fine particles and the activity of the particles was measured

[3] Activity measured/activity added ·100

[4] Apparent activity measured for each of the construct shapes

The apparent activity of Printzymes with 4 mm thickness was much lower than the maximum activity: the cylinder shapes showed a 99 - 120 fold reduction in apparent activity whereas torus shapes showed 46 - 48 fold reductions in apparent activity (Table 2). Hence, the effectiveness factor, which is the ratio between enzymatic activity monitored in the immobilised state compared to free solution (Eq. 2), was close to zero for the cylinder shape (0.008 and 0.01 for 0.4-20 and 4-20 cylinder shapes, respectively). This indicates that the substrate cannot penetrate through the device and thus reaction rate is limited to the outer layer of the system (Doran, 2012). The apparent activities of the 3 mm thickness cylinder constructs were slightly higher than the 4 mm counterparts, demonstrated by effectiveness factors of 0.03 - 0.07. However, reducing the thickness to 3 mm affected the system's structural integrity, which was lost upon storage (data not shown). Hence, the 4 mm thickness was selected for progression to further experimentation.

The torus designs of both Printzyme 0.4-20 and 4-20 at 4 mm thickness had higher activities than their cylinder counterparts, due to an increased number of exposed laccase active sites arising from an increased surface area. The torus forms of Prinzyme 0.4-20 and 4-20 had 159 % and 103 % higher relative activities than the cylinder designs. In practice, the geometry of Printzymes will be adapted to suit their application. For example, torus shapes may be best suited to line

tubes or pipes, whereas more complex morphologies may be suited to other applications requiring bespoke designs. In any case, this data demonstrates that recognition of device surface area is a key design consideration with direct implications for Printzyme activity.

3.3 Optimal pH and temperature for Printzyme activity

Figure 3a shows how free laccase and Printzyme activity changed in conditions of varying pH (pH 3.0 - 6.0). For both Printzyme formulations and free laccase, pH and enzyme activity showed an inverse correlation, i.e., with increasing pH, enzymatic activity decreased. Printzyme 0.4-20, Printzyme 4-20, and free laccase all demonstrated optimal activity at pH 3.0. This observation is in accordance with the literature, in which pH 3.0 has been shown as the optimal pH for free laccase and for laccase immobilised onto fumed silica nanoparticles (Arca-Ramos et al., 2016; Vera and Rivas, 2017). Depending on the locations and types of water affected, drugs will require removal from aqueous systems of varying pH and temperatures. In the United States, rainfall can vary from lower than pH 4.3 to over pH 6.0 (National Trends Network, 2002). Soil pH can vary widely from pH 2.0 - 9.0, depending on solubilised minerals, thus affecting the pH of groundwater (Thomas, 1996). Wastewater pH will vary according to the processes and environments it has been exposed to. These data show that Printzyme performs best at acidic pH, which should be considered in its future applications.

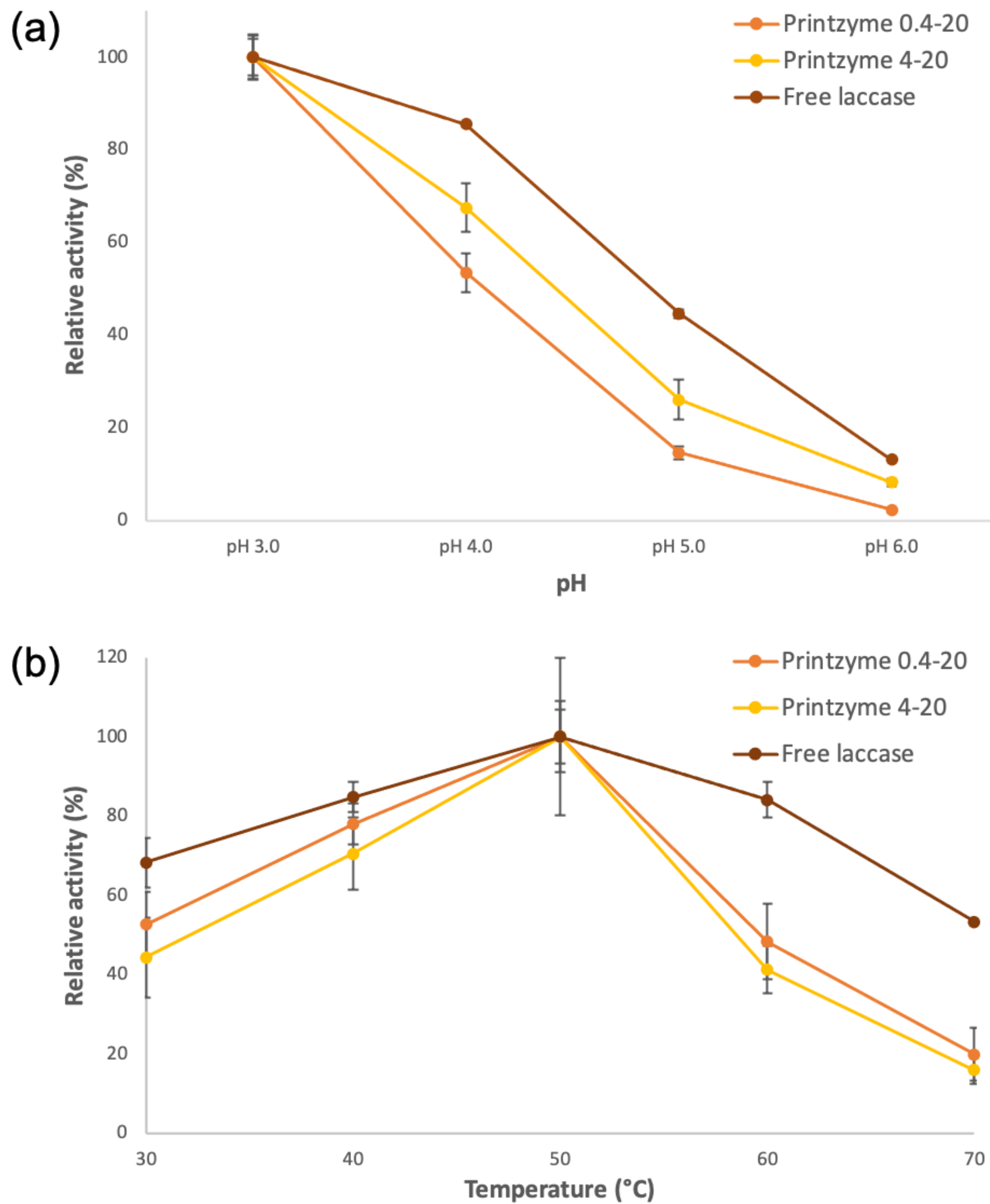


Figure 3. Effect of (a) pH and (b) temperature on the activity of free laccase, Printzyme 0.4-20 and Printzyme 4-20. Data values represent mean ± SD, which are not seen in some data points as they are smaller than the symbols.

With regards to temperature, enzyme activity in both Printzyme formulations and free laccase demonstrated a bell-shaped curve, where enzyme activity peaked for all three at 50 °C (Figure

3b). These results concur with other studies, in which 50 °C was determined as the optimal temperature for laccase sourced from *T. versicolor* (Litwińska et al., 2019; Šnajdr and Baldrian, 2007; Vera and Rivas, 2017). The increase and subsequent decline in enzyme activity is unsurprising, as it follows the inherent behaviour of most biological enzymes. With increasing temperature, the energy in a system increases with the rate of enzyme catalysis, until an optimum temperature is reached; above which irreversible enzyme denaturation begins (Peterson et al., 2007). Applications of Printzyme should consider this activity to temperature relationship, potentially avoiding use above 50 °C, as irreversible laccase denaturation will impact the reusability of devices.

In both the pH and temperature studies (Figures 3a and 3b), free laccase demonstrated higher activity than when immobilised in Printzyme. This is likely because in solubilised form, all laccase active sites are freely accessible, whereas in Printzyme enzyme immobilisation leads to a certain degree of steric inhibition. Whilst Printzymes do achieve lower activity in aqueous solutions compared to free laccase, they still retain substantial activity. Moreover, both Printzyme formulations demonstrated similar relationships to pH and temperature as free laccase, indicating that direct immobilisation of laccase into PEGDA hydrogels does not alter the biocatalytic mechanism. In addition, immobilisation affords benefits in storability and reusability that free enzyme in solution can not accomplish (Fernandez-Fernandez et al., 2013). Enzyme entrapment within functional systems enhances sustainability and cost-effectivness as expensive enzymes are not lost with every use. This is especially important for settings with limited storage, budgets, or access to deliveries (e.g., in remote locations).

3.3 Effect of pH and temperature on Printzyme stability

Interestingly, Figure 4 shows that whilst free laccase and Printzyme activity is optimal at acidic pH (as shown in Figure 3a), enzyme activity suffers during extended exposure to acidic conditions. During the functional use of Printzyme it will likely be subjected to aqueous

environments of varying pH and temperatures for extended periods. This data highlights that Printzyme should be stored around neutral pH, and only exposed to acidic media for short time periods, to retain maximal activity and reusability. Figure 4b demonstrates that immobilisation of laccase in Printzyme 0.4-20 protects it from degradation, with minimal loss of enzyme activity at pH 4.0 - 8.0 over 24 hours. In comparison, free laccase is observed to lose activity in acidic environments (pH 3.0 and 4.0) when present in solutions for extended periods (Figure 4a). Such deactivation of free laccase has been noted in previous studies (Kurniawati and Nicell, 2008; Rancano et al., 2003). This protective effect of Printzyme could be attributed to the hydrogel matrix resulting in restricted conformational mobility of laccase (Li et al., 2015; Lloret et al., 2011).

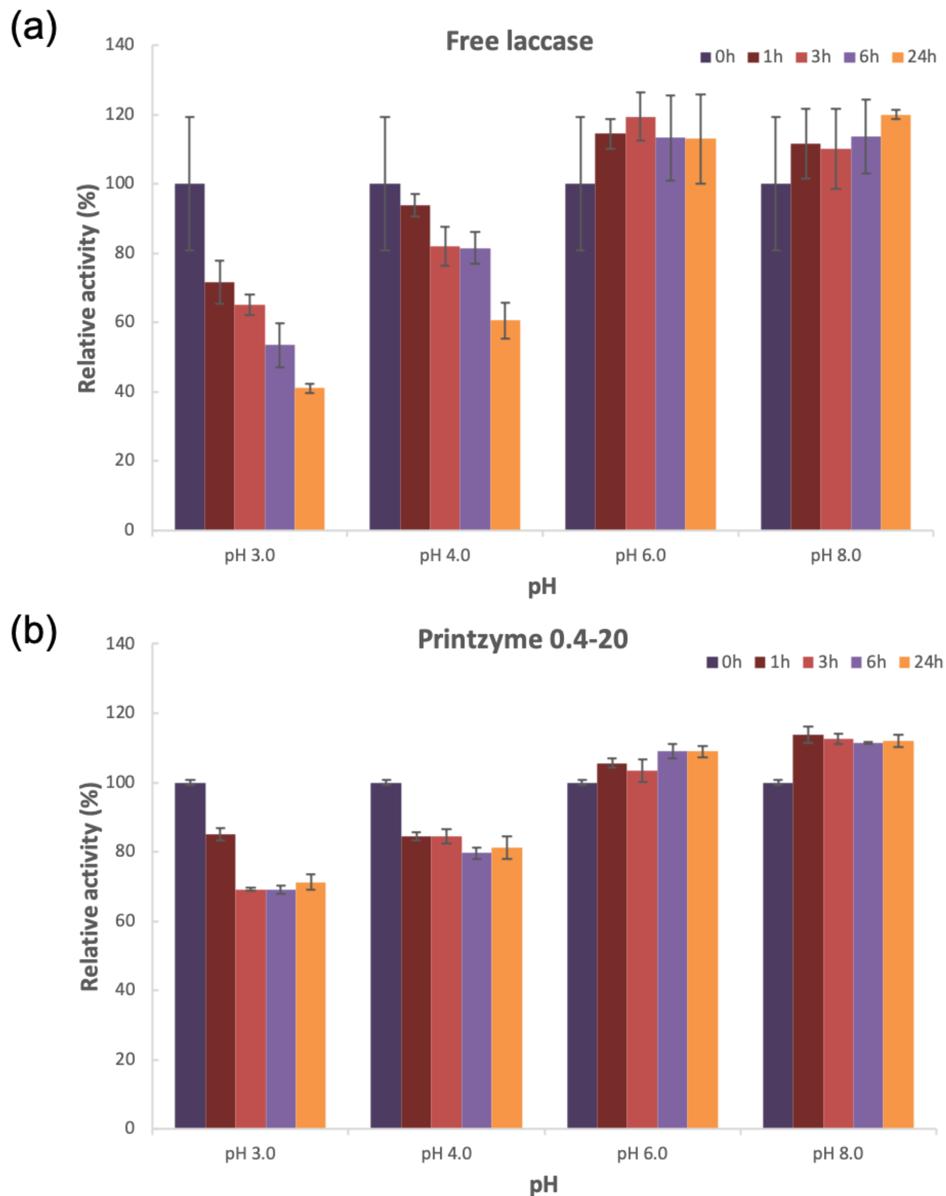


Figure 4. pH stability of (a) free laccase and (b) Printzyme 0.4-20 after 1 hour, 3 hours, 6 hours and 24 hours of incubation in various pH values at room temperature. Data values represent mean ± SD.

The protective effect of Printzyme 0.4-20 was also observed in the thermostability study, where devices were incubated at 40 °C for 24 hours (Figure 5). At 40 °C, free laccase lost its activity from 6 hours onwards, whereas laccase in Printzyme did not experience substantial denaturation.

This improved thermostability has also been observed with laccase-entrapped nanoparticles, though these systems can be difficult to prepare compared to Printzyme's simultaneous print-and-immobilise manufacture (Arca-Ramos et al., 2016). For both free laccase and Printzyme, biocatalytic activity decreased when maintained in aqueous conditions of 50 °C and over (however Printzyme did exert a protective effect for the first 3 hours). This progressive loss of enzyme activity is most likely due to thermal denaturation and loss of laccase tertiary structure, and indicates that Printzyme should not be stored at temperatures exceeding 40 °C (Kurniawati and Nicell, 2008). The ability of Printzyme to protect laccase activity under conditions of varying pH and temperature may go some way to compensating towards its lower relativity activities in certain conditions when compared to free laccase (Figure 3).

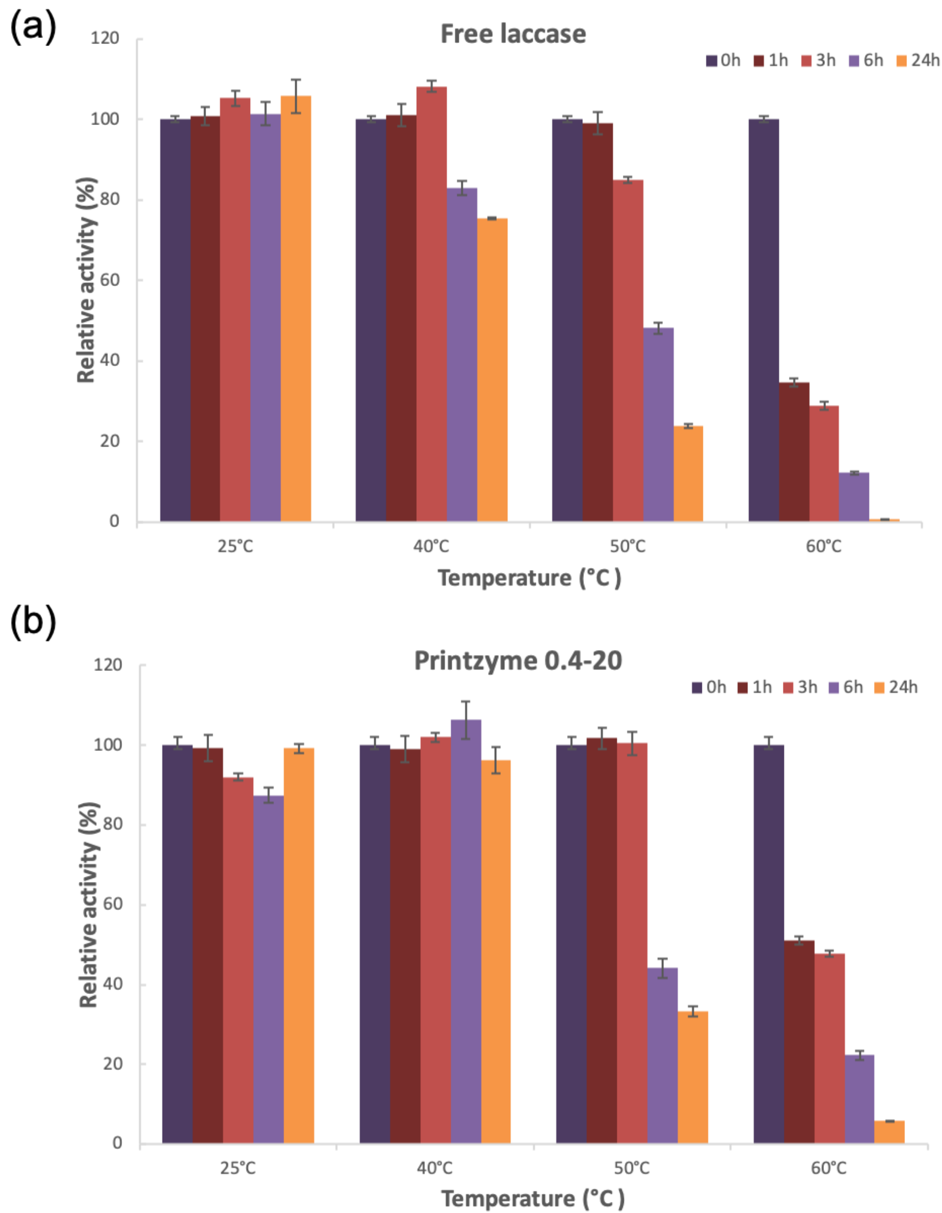


Figure 5. Thermal stability of (a) free laccase and (b) Printzyme 0.4-20 after 1 hour, 3 hours, 6 hours and 24 hours of incubation in citric-phosphate buffer (pH 6.0) at different temperature values (25 °C, 40 °C, 50 °C and 60 °C). Data values represent mean ± SD.

3.4 Reusability of Printzymes

Figure 6 shows the activity of laccase over 18 consecutive uses of 5 minutes each when immobilised in Printzyme 0.4-20 and 4-20. A duration of 5 minutes was chosen as this is the time required for oxidation of the test substrate, ABTS. Clearly, both Printzyme formulations retained good activity despite multiple cycles of use, with Printzyme 4-20 demonstrating the best stability with its higher enzyme content. After 5 isolated uses, Printzymes 0.4-20 and 4-20 retained 85.4 ± 2.6 % and 87.0 ± 2.5 % of their initial activities, respectively. The enzymatic activity of both Printzymes remained almost constant, achieving 83.0% and 107.0% relative activities, respectively, after 18 consecutive cycles. Both Printzymes achieved superior reusability compared with other immobilisation methods of laccase from *Trametes versicolor*, including iron oxide nanoparticles 33% (Iriarte-Mesa et al., 2019), chitosan macrobeads (Apriceno et al., 2017), magnetic mesoporous silica spheres (Zhu et al., 2007), poly(glycidyl methacrylate)-based microspheres (Vera and Rivas, 2017), and agar-agar, polyacrylamide, and gelatin structures (Asgher et al., 2017). During muitlple cycles of use, the Printzyme shape was maintained with a noticeable colour change to green due to adsorption of ABTS transformation products, a process that did not affect enzyme activity (Figure S1, Supplementary Material). As mentioned, a key benefit of Printzyme is its immobilisation of laccase, as this allows enzyme recovery and repeated use not possible with free enzyme in solution. These data highlight the concept's cost-effectiveness, sustainability, and fit for purpose.

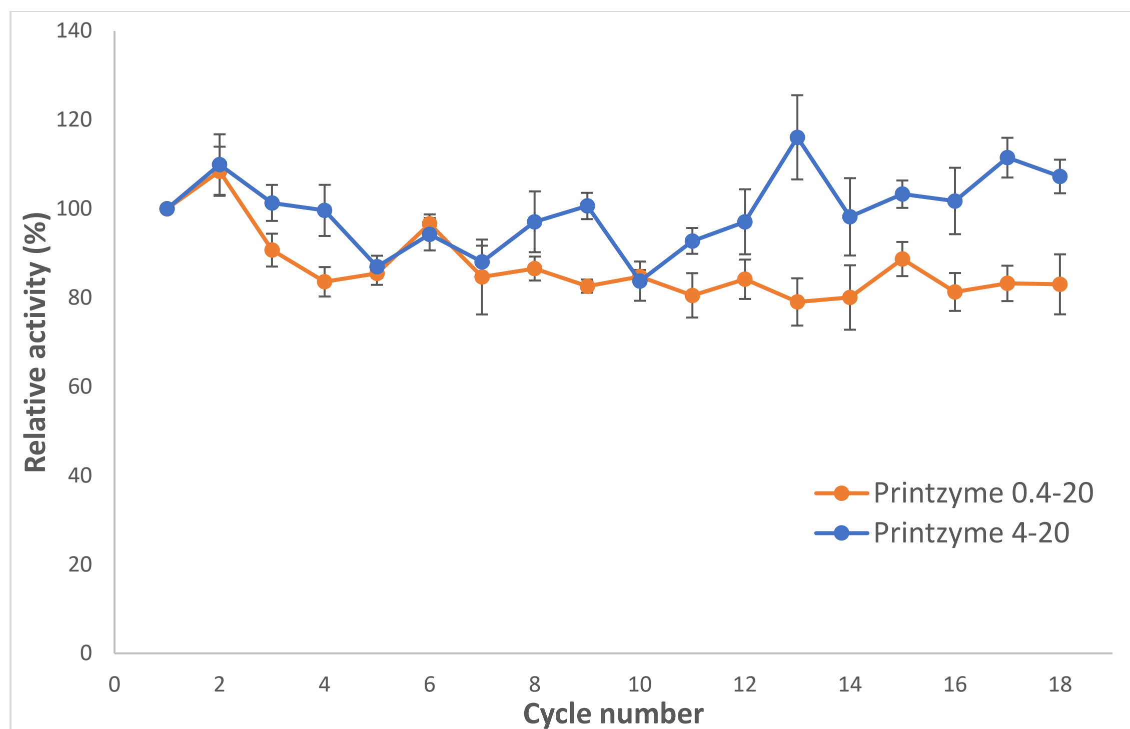


Figure 6. Reusability of Printzyme 0.4-20 and Printzyme 4-20, demonstrated by retention of laccase relative activity. Data values represent mean ± SD.

3.5 Removal of pharmaceuticals using Printzyme

Figure 7 shows the degradation of diclofenac and ethinylestradiol over 48 hours, in the presence of Printzyme and free laccase. Clearly, both Printzyme shapes and free laccase achieved significant degradation of both drugs compared to controls from 2 hours onwards ($p<0.05$). Both drugs are common water pollutants on a global scale, and as such are present on the European watch list for environmental micropollutants (Barbosa et al., 2016). Printzyme 4-20 was selected for this investigation, due to its higher resolution, laccase content, and resusability compared to other formulations tested in the study. The fact that the formulation is 80 % water is an additional benefit, as this reduces the manufacture cost per device and decreases its carbon footprint (due to lower consumable PEGDA content).

Figure 7a shows that the torus form of Printzyme achieved faster and more complete removal of diclofenac compared to the cylinder, tablet-shaped, device ($p<0.05$). This is unsurprising, as Section 3.2 showed that laccase activity was enhanced by the torus' higher surface area. Using the torus Printzyme, 50 % of diclofenac was removed after 4 hours, and after 24 hours it had achieved near complete degradation of the anti-inflammatory. Regarding the cylinder Printzyme, it achieved 50 % degration after 8 hours and after 48 hours the removal of diclofenac has been nearly completed (>90 %). During the experiment, the degradative action of immobilised laccase within Printzyme was made visually clear, as the originally colourless solution of diclofenac gradually became dark yellow. This is likely due to the accumulation of a known diclofenac degradation product, 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol (Marco-Urrea et al., 2010). Surprisingly, the initial removal rates with free laccase in solution were lower than those achieved with both Printzyme configurations. This implies that the effectiveness factor (Eq. 2) reached values higher than one (1.55 and 1.11 for torus and cylinder, respectively, calculated for the removal rates at 8 hours). Effectiveness factor generally lies between 0 and 1, but sometimes can be higher than one due to non-isothermal operation, because of the stabilisation of the encapsulated enzyme related to the free one or because of inhibitory effects (Chaplin and Bucke, 1990). Here, the most plausible reason is the inhibition caused by high diclofenac concentrations, as reported by Lonappan et al. (Lonappan et al., 2017). These authors observed that at 5 mg/L concentration the kinetics of *T. versicolor* laccase shifted from first order to zero order, confirming substrate inhibition. In this work, Printzyme may have increased observed degradation rates by reducing the concentration of the substrate to non-inhibitory concentrations. Hence, Printzyme represents the more economic and sustainable option not only due to its reusability but also because it was more effective than the free enzyme in degrading diclofenac in the short term. Other pollutants reported as inhibitors of *T. versicolor* laccase include dyes (Stoilova et al., 2010) or phenolics from the olive oil industry (Canfora et al., 2008).

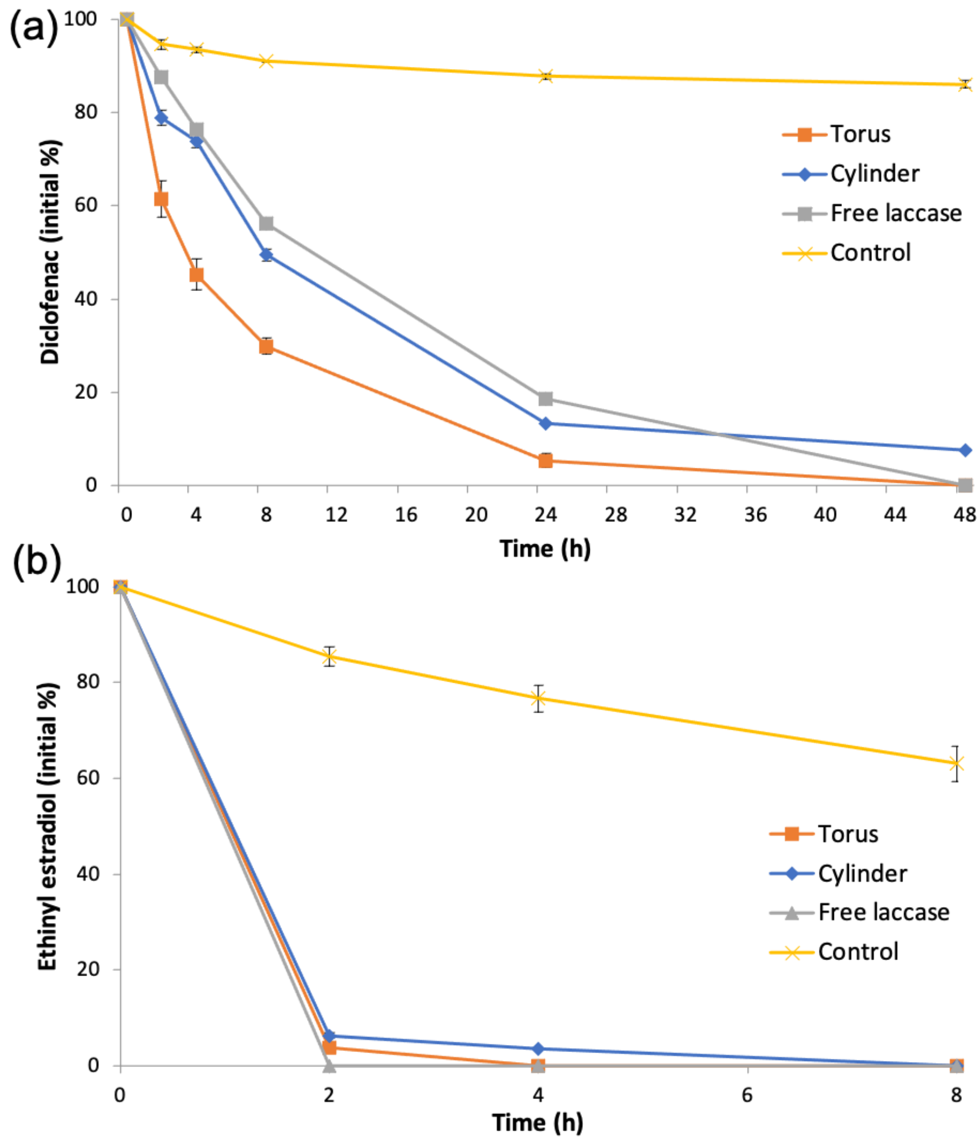


Figure 7. Effect of torus and cylinder Printzyme 4-20 on removal of (a) diclofenac and (b) ethinyl estradiol. Control uses Printzyme lacking laccase. Data values represent mean ± SD, which are not seen in some data points as they are smaller than the symbols.

Figure 7b shows how both Printzyme designs achieved near complete removal of solubilised ethinylestradiol after just 2 hours ($p < 0.05$ compared to control). Similar results have been reported in previous studies in which more than 80 % of ethinyl estradiol has been removed following just 1 hour treatment of laccase from *T. versicolor* (Auriol et al., 2008). Noticeably, the

control experiment showed removal of the oestrogen over the 48 hours. This is likely due to adsorption of the drug onto the laccase-free Printzyme control. In fact, recoveries of 81 % were obtained after extraction of the Printzyme in methanol, indicating that the drug was adsorbed onto the control Printzyme, demonstrating an additional mechanism of drug removal for the device (Lloret et al., 2011; Lloret et al., 2012). Despite this, the data indicates that Printzyme's removal of ethinylestradiol was mainly due to the action of the immobilised laccase. The effectiveness factor (Eq. 2) for the estrogen removal was close to one in both cases (0.92 and 0.95 for cylinder and torus, respectively, at 2 h), proving that mass transfer had a negligible effect on the overall reaction rate. After the experiment, all the Printyzmes were recovered from the flasks and an increase in size was observed due to swelling of the hydrogel (Figure S2, Supplementary Material).

Figure 7 demonstrates how Printzyme's removal capacity differed for the two investigated drugs. Laccase is a copper-containing oxidase that catalyses oxidation reactions through single electron transfer; it is known to show high activity against phenolic compounds (Jeon et al., 2012; Rodríguez-Delgado et al., 2015). Ethinylestradiol contains a phenolic moiety within its chemical structure whereas diclofenac does not, thus explaining why Printzyme removed ethinylestradiol faster than diclofenac. Phenol groups are common in medicinal chemistry, and are present in a wide variety of drugs, from the anti-Parkinson's drug entacapone, to the antimuscarinic tolterodine, to ivacaftor for cystic fibrosis. This feature of laccase should be considered in Printzyme's applications. Particularly when the substrate can cause laccase inhibition, the use of Printzyme is highly recommendable, since shorter degradation times may be required compared to free enzyme. If fast removal (under 24 hours) of non-phenolic pharmaceuticals is required, then a mediator may be needed in the Printzyme formulation. Laccase mediators, including syringaldehyde, acetosyringone, vanillin, and p-coumaric acid have been previously shown to enhance the degradation of non-phenolic drugs (Becker et al., 2016).

Although this work has used drug concentrations relevant to sites with high pharmaceutical burden, such as hospitals and pharmaceutical production sites, where concentrations can reach the mg/L range, it should be highlighted that laccase has been successfully used to treat micropollutants in municipal wastewaters (Spina et al., 2020). The ability of Printzymes to challenge micropollutants in municipal wastewaters and/or surface waters is a intended feature of future work. In general, Printzyme provides a reusable, customisable, cost-efficient, and sustainable way of effectively removing drugs from concentrated aqueous systems. It represents a flexible platform that can be used to immobilise a wide variety of enzymes for different specialist applications. Importantly, Printzyme technology has been shown to protect enzymes from deactivation at extremes of pH and temperature, making it a versatile tool for industrial processes including the removal of drugs from water.

Conclusions

In this study, we immobilised laccase sourced from *T. versicolor* for the first time in a PEGDA hydrogel via SLA printing, with the goal of developing a customisable, economic, and effective way of removing drugs from water: a key global issue. During formulation tests, it was determined that optimal SLA resins incorporated PEGDA and water in a 20:80 ratio. Successful design and 3D printing of devices produced Printzymes with homogenously distributed 0.4% and 4% w/w laccase, in torus and cylinder shapes. Post-printing analysis of enzyme activity confirmed that higher resin laccase concentrations led to an increase in activity. By studying Printzyme activity at extremes of pH and temperature, it was confirmed that Printzymes function best in acidic solutions of around 50 °C, however to retain maximal reusability they require storage at neutral pH and less than 40 °C. With regards to reusability, Printzymes were shown to retain good laccase activity for at least 18 isolated uses; demonstrating their superiority over free laccase in solution. Further, Printzyme could successfully reduce the concentrations of two drugs, diclofenac and ethinylestradiol, in aqueous solution. The devices removed over 80 % of dissolved diclofenac within 24 hours, and 95 % of ethinylestradiol within just 2 hours, being more efficient the torus shape, because of the higher surface to volume ratio. The effectiveness factor for the estrogen removal was close to one (0.95 for torus Printzyme), demonstrating that substrate diffusion across the Printzyme did not limit the transformation. Regarding diclofenac, the device provided higher initial removal rates than the free enzyme, with effectiveness factor of 1.55 (torus Printzyme), emphasizing the interest of using Printzyme for the transformation of substrates which can inhibit laccase. This work highlights the potential of SLA 3D printing as a streamlined approach to entrap enzymes under mild conditions. Our results present a significantly effective and sustainable approach to removal of pharmaceutical contaminants from aqueous systems and provide implications for the development of bioremediation.

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