3D printed UV/VIS detection systems constructed from transparent filaments and immobilised enzymes

Elaheh Sirjani¹, Maciej Migas¹ Peter J. Cragg¹ and Marcus K. Dymond^{1*}

¹Division of Chemistry, School of Pharmacy and Biological Sciences, University of Brighton, Lewes Road, Brighton. BN2 4GL.

*Email: M.Dymond@brighton.ac.uk

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Abstract

Transparent materials for fused filament fabrication printers are widely available and may be useful for constructing 3D printed devices with applications in UV/VIS spectroscopy. In this study, colourless polylactic acid, HD Glass and T-Glase were evaluated as construction materials for biochemical sensors, which contain immobilised enzymes, for analysis by UV/VIS spectrophotometry. Experiments were conducted on both the native 3D print and after coating with XTC-3D[®], a transparent epoxy resin used to improve optical transparency of 3D prints. A combination of HD Glass or T-Glase with XTC-3D[®], gave the highest transparency, probably due to the similar refractive indices of these materials. Individual enzymes were immobilised within the 3D prints by coupling the enzymes to tosyl-activated magnetic beads and attracted to the print surface by magnets embedded in the 3D print. A transparent 3D printed device was demonstrated using enzymatic assays of lactose and glucose. This device can be configured to contain up to three separate reaction chambers and features a separate colorimetric analysis chamber. Further studies showed that enzyme assays performed in these 3D printed devices are reproducible, accurate and of comparable sensitivity to the same assays performed in polystyrene cuvettes.

1.0 Introduction

Developing the capability to 3D print optically transparent materials has a broad range of applications but one topical area that would benefit is biosensor development. For example, 3D printed biosensors that enable enzyme-catalysed reactions to be performed and quantified spectroscopically have the potential to make biosensor development accessible to a wider range of end-users. These biofunctionalized 3D printed devices might be constructed for bespoke, low-throughput applications, or as part of a prototype workflow for high-throughput injection moulded designs. Recent progress in this area has been driven by the decreasing cost of fused filament fabrication (FFF) 3D printers, which means additive manufacturing techniques are becoming increasingly available to researchers in the chemical and biological sciences. One of the advantages of FFF 3D printers is that they can print a wider range of materials than 3D printers that employ UV curable polymers, which expands the range of potential 3D printing applications.

In the chemical and biological sciences there is already a significant number of examples of published 3D printed devices [1–6], including reactionware [7–10] and microfluidic devices [11–13], together with accounts of spectroscopic applications [14–25]. There are, however, several challenges that need to be addressed before 3D printed devices can be routinely constructed for spectrophotometric applications, particularly for FFF printers. These challenges stem from the additive manufacturing technique itself. FFF builds the 3D form from a series of thin extruded plastic layers and when transparent filaments are used each extruded layer acts like a small lens, scattering light, resulting in low light transmission to the photodetector. Another disadvantage with the FFF method of 3D printing is that it leaves air pockets and other print imperfections [26] in the final 3D print. These faults may also cause light scattering and make it difficult to construct watertight 3D prints, a problem that is compounded when applications require flowing water and elevated pressure. It is possible to make the individual layers of the FFF 3D print adhere more tightly by immersing the final print in a solvent, as recently demonstrated [14]. However, this solvent immersion technique

is not suitable for spectrophotometric methods, since the transparency of the final print is adversely affected.

A number of other design features are also important to maximise the utility of 3D printed biosensors [17]. For example, watertight devices constructed in a single-step process, with low print fail rates, are important for efficiency. Transparent 3D printed parts with a wide working range of wavelengths across the UV/VIS spectrum are important to maximise device compatibility with spectroscopic molecular probes/assays. Restricting the final dimensions of the design so devices can be analysed in a UV/VIS spectrophotometer, using a standard cuvette holder, lowers the take-up barrier for new adopters of the technology. Furthermore, many analytical assays contain multiple enzymes that require several consecutive reactions to be performed prior to substrate quantification [27,28], therefore 3D prints that contain immobilised enzymes in one or more reaction chambers and feature a separate analysis chamber for colorimetric detection are desirable to maximise compatibility with a wide range of assay systems. Typically, such enzyme assays require users to transfer reaction solutions into a cuvette prior to analysis, however the versatility of 3D printing opens up the possibility of transferring solutions between reaction and analysis chambers simply by rotating the device. A final advantage of using immobilised enzymes is that rotation of the device removes the enzymes from the reaction mixture prior to analysis. This prevents chemical reactions from occurring past the allotted assay time, which may improve assay accuracy, and may also allow devices to be used multiple times.

Enzyme based assays in 3D printed devices have previously been explored [29,30] and often use glucose or lactose detection systems because the assays are robust. They can be performed over a range of temperatures and the individual enzymes involved are easily immobilised [31]. Enzymatic methods to detect glucose are usually based on the glucose oxidase protein (GOD) [28], which oxidises β -D-glucose in the presence of oxygen to β -D-glucono- δ -lactone and H_2O_2 [32]. Subsequently, H_2O_2 oxidises a chromogenic substrate in the presence of horse radish peroxidase (HRP) causing a colour change that is monitored with a UV/VIS spectrophotometer. A number of chromogenic substrates can be used such as *o*-dianisidine [33], which forms a

quinoneimine dye with $\lambda_{\max}$ at 500 nm [34], or Amplex Red ($\lambda_{\max}$ at 570 nm). Lactose detection [27] systems build in an extra enzymatic step, hydrolysing lactose into D-galactose and β -D-glucose, with β -galactosidase (β -Gal). Subsequently β -D-glucose, or alternatively D-galactose, are detected. This assay system has found widespread use in biosensing applications [35].

Herein, using enzymatic assays of glucose and lactose as a test system, a series of versatile 3D printed bioanalytical devices is presented. These devices contain up to three enzymes immobilised in different reaction compartments within the 3D print, as well as a separate analysis chamber. The final device fits within a standard cuvette holder for analysis by UV/VIS spectroscopy and the reaction solution is transferred between compartments by rotating the device in different directions. In parallel, several transparent 3D printable filaments and XTC-3D[®] (a transparent epoxy resin, which improves light transmission, added post-production) were evaluated for their utility in applications relating to UV/VIS spectroscopy. The filaments analysed were colourless polylactic acid (PLA), HD Glass (derived from polyethylene terephthalate glycol) and T-Glase (derived from polyethylene terephthalate). These filaments were selected because amateur model makers anecdotally report good results with them in combination with XTC-3D[®], which fills gaps between the extruded filament layers, resulting in a more transparent print. The compatibility of the filaments with enzyme assays of glucose or lactose is determined. Using different combinations of enzymes, chromogenic substrates, the robustness and versatility of FFF 3D printing as a tool for fabricating biofunctionalized, transparent spectroscopic equipment is evaluated.

2.0 Materials and methods

UV/VIS spectroscopy was performed on a Varian Cary 50 Scan UV-Visible Spectrophotometer. 3D Printing was performed on a Makerbot Mini 3D printer. 3,5-Dinitrosalicylic acid, phenol, potassium sodium tartrate tetrahydrate, glucose oxidase (*Aspergillus niger* Type VII, E.C.1.1.3.4), horseradish peroxidase (Type II, E.C.1.11.1.7), β -Galactosidase (*Escherichia coli* Grade VIII, E.C.3.2.1.23), D-(+)-glucose and D-lactose monohydrate, sodium phosphate, ammonium sulfate and Tween 20 were purchased from Sigma-Aldrich, UK. Sodium hydroxide, Dynabeads™ M280 (tosyl-activated) and sodium sulfite (anhydrous) were purchased from Fisher Scientific UK. Fluorescein sodium salt was purchased from Hopkin & Williams UK and deionised water (Veolia Water ELGA PURELAB flex) was used throughout.

Transparent PLA, HD Glass, and T-Glase 3D printing filaments were purchased from MakerBot, Formfutura, and Taulman3D, respectively. 3D Prints were drawn in Autodesk® 123D® Design software, exported as .STL files and sliced in Makerbot Desktop. Nickel plated neodymium magnets (6 mm diameter by 1 mm height) were purchased from First4magnets (Tuxford, Nottinghamshire, UK).

2.1 3D Printing settings

Colourless PLA, HD Glass and T-Glase were printed at 215°C, 220°C and 235°C with a single shell, 100% infill and a raft. The Makerbot Mini does not have a heated build platform however excellent adherence for all materials was obtained by printing on double sided tape, at room temperature. All other 3D printer settings were kept as standard for PLA on the Makerbot Mini 3D printer except the extruded print layer height, which was varied as a part of the experiments. A full list of the standard PLA 3D print settings is provided as supporting information.

2.2 Characterising the optical properties of colourless PLA, HD Glass and T-Glase for applications in UV/VIS spectroscopy

The optical properties of transparent FFF printer filaments were evaluated using a 3D printed cuvette with final measurements of 46.0 mm x 10.8 mm x 10.8 mm (H x W x D) with 0.4 mm wall thickness and 2 mm base. A wall thickness of 0.4 mm corresponds to a single thread of extruded plastic, maximising optical transparency (see Figure S1). All cuvettes were 3D printed with the settings described in section 2.1 with the exception that prints were made at 0.1 mm, 0.2 mm and 0.3 mm layer heights for each material.

UV/VIS spectroscopy was performed in triplicate on empty cuvettes made from PLA, HD Glass, and T-Glase, before and after the application of XTC-3D[®] coating. XTC-3D[®] (prepared according to the manufacturer instructions) was brushed onto the internal and external printed surfaces of the 3D printed cuvette. Treated cuvettes were then left in upright position to dry for >3.5 hr at room temperature. Empty cuvettes were placed into the UV/VIS spectrophotometer and scanned from 200-800 nm at 5 nm intervals. To ensure direct comparability of the results for the different filaments, the instrument was blanked with an empty polystyrene cuvette, before each analysis.

2.3 Glucose quantification assay

A glucose absorption assay [36] utilising 3,5-dinitrosalicylic acid (DNS) (1% w/v) and Rochelle salt was used to test the linear response and variability between 3D printed cuvettes. The assay was validated by performing in triplicate on glucose standards using disposable polystyrene cuvettes. Absorbance measurements were recorded at 575 nm. Before each analysis the instrument was blanked with an empty polystyrene cuvette, to ensure direct comparability of the results for the different filaments.

DNS (1% w/v) reagent was prepared by dissolving DNS (5.0 g), phenol (1.0 g), sodium sulphite (0.25 g) and sodium hydroxide (5.0 g) into 500 ml water, shaking vigorously until the contents were fully dissolved. Rochelle salt (potassium sodium

tartrate solution (40 % w/v)) was prepared alongside solutions of glucose in water, at 0.1, 0.25, 0.50, 0.75 and 1.0 mg/ ml. Glucose was quantified by mixing DNS (6 ml, 1% w/v) and glucose solution (6 ml) in a boiling tube. After heating in hot water for 5 minutes (or until a colour change to dark red/brown was observed), 1 ml of 40% potassium sodium tartrate solution was added to the sample and mixed well. The mixture was cooled to room temperature before transferring to a cuvette for UV/VIS analysis.

2.4 Protein conjugation to tosyl-activated magnetic beads (Dynabeads™ M280)

GOD, HRP and β -Gal were coupled to tosyl-activated Dynabeads using the standard manufacturer's protocol, summarized briefly below, to give GODDynabeads, HRPDynabeads and β -GalDynabeads.

The following buffers were prepared (as per the manufacturers standard protocol),

Buffer A: Sodium phosphate buffer (0.1M), pH 7.4.

Buffer B: Ammonium sulphate buffer (3M), pH 7.4.

Buffer C: Sodium phosphate buffer with 0.5% Tween 20 (w/v), pH 7.4.

Buffer D: Sodium phosphate buffer with 0.1% Tween 20 (w/v), pH 7.4.

Dynabeads™ (used as supplied) were resuspended by vortexing before 335 μ l of beads were transferred to a 1 ml microcentrifuge tube. Buffer A (335 μ l) was added and the mixture was mixed by vortexing. A strong magnet was then applied to separate the beads and the supernatant solution was removed with a pipette. This washing step was repeated before the Dynabeads™ were resuspended in 250 μ l Buffer A, containing either β -Gal, GOD or HRP proteins at 8 mg/ ml. Buffer B (100 μ l) was then added and the solution was mixed by gentle pipetting and incubated at 37°C for 12–18 hours.

After incubation Dynabeads™ were separated using a strong magnet and the supernatant solution was removed. 1 ml of Buffer C was added, and samples were mixed and incubated at 37°C for 1 hour. After separation with a strong magnet the supernatant was removed, and 1 ml Buffer D was added. These, protein coupled

Dynabeads™, were washed two further times with Buffer D (1 ml), before resuspending in 480 µl of Buffer D and storing in a fridge (8°C).

Protein-coupled Dynabeads™ were incorporated into assays after resuspension by vortexing, then the required volume of protein coupled Dynabeads™ (typically 100 µl) was transferred into a new vial. Dynabeads™ were separated with a strong magnet and the supernatant was removed with a pipette, before resuspension in Buffer C (typically 100 µl) and subsequent addition to the assay or 3D printed device.

2.5 Transparent 3D printed devices

3D Printed bioanalytical devices containing either one, two or three reaction chambers were printed in HD Glass using the settings described in section 2.1 and at 0.1 mm layer height. Individual diagrams and STL files for each design are included as supporting information. Beneath the floor of each reaction chamber, a void (or multiple voids, depending on the design) was included to accommodate a 6 mm diameter magnet. Part way through the printing process, printing was paused and, neodymium magnets were placed in each of the voids, gluing in place with Gorilla™ superglue adhesive, if necessary. Figure S2 shows the magnets in place during printing, after completion the exterior of the 3D printed device was coated with XTC-3D® and dried for > 3.5 hours.

2.6 A coupled enzymatic assay of lactose performed in a transparent 3D printed device with one reaction chamber

Studies utilising 3D printed bioanalytical devices with a single reaction compartment containing three separate magnets were set up by individually adding β-GalDynabead (50 µl), GODDynabead (50 µl) and HRPDynabead (50 µl) solutions (in Buffer C) to the area above a different magnet in the device and leaving to rest for 30 minutes. Next 150 µl of lactose solution, at the appropriate concentration, and 200 µl of Amplex Red (3 mM) were added to the reaction compartment. The mixture was incubated at 37°C for 1 hour. At the allotted time, the solution mixture was tipped into

the analysis chamber and the absorbance was measured by UV/VIS spectroscopy (570 nm). Final lactose concentrations studied were 0.5, 1.2, 2.5, 3.7, 5.0 and 6.3 mM. Each study was performed in triplicate. Before each measurement an empty 3D printed device of the same specification as those used in the assay was used to zero the UV/vis spectrophotometer.

2.7 A coupled enzymatic assay of glucose performed in a transparent 3D printed device with two reaction chambers

Reactions were performed stepwise in a 3D printed bioanalytical device with two reaction chambers. Initially GODDynabeads (35 μ l) were added to the first reaction chamber and allowed to settle on the magnet. Next 500 μ l of Buffer B, containing the appropriate concentration of glucose solution, and *o*-dianisidine (0.17 mM) were added. The mixture was incubated at 37°C for 30 min. Then HRPDynabeads (35 μ l) were added to reaction chamber two and the device was rotated to displace reaction mixture into chamber two, incubating at 37°C for a further 30 min. The final reaction products were transferred into the analysis chamber by rotating the device and the 3D print was placed inside the cuvette holder of a UV/VIS spectrophotometer. Absorbance measurements (500 nm) were recorded immediately. Final glucose concentrations studied were 0.1, 2.5, 5.0, 7.5, 10.0 and 12.0 mM. Each concentration was analyzed in triplicate using an empty 3D printed bioanalytical device to zero the spectrophotometer.

Control studies were performed by mixing GOD and HRPDynabead solutions with the reagents, at identical concentrations to those presented above, in a single polystyrene cuvette, incubating at 37°C for 30 min.

2.8 A coupled enzymatic assay of lactose performed in a transparent 3D printed device with three reaction chambers

Reactions were performed stepwise in a 3D printed bioanalytical device with three reaction chambers, initially β -GalDynabeads (50 μ l), followed by the appropriate

concentration of lactose solution (150 μ l) and Amplex Red (3 mM, 200 μ l), were added to reaction chamber one and incubated at 37°C for 30 min. Next, GODDynabeads (50 μ l) were added to the second reaction chamber and after 1 min the solution inside of the first chamber was displaced into the second chamber by rotating the 3D printed device. The reaction mixture in chamber two was incubated at 37°C for 30 min. Then HRPDynabeads (50 μ l) were added to reaction chamber three and the device was rotated to displace reaction mixture chamber three, which was incubated at 37°C for a further 30 min. The final reaction products were transferred into the analysis chamber by rotating the device. The 3D printed bioanalytic was placed inside the cuvette holder of a UV/VIS spectrophotometer and absorbance measurements (570 nm) were recorded immediately. Final lactose concentrations studied were 0.5, 1.2, 2.5, 3.7, 5.0 and 6.3 mM, each concentration was analyzed in triplicate, using an empty 3D printed bioanalytical device to zero the spectrophotometer.

3.0 Results

Before constructing and testing complex 3D prints, the performance of the transparent filaments was evaluated for potential applications in UV/VIS spectroscopy. Light transmission, the range of working wavelengths, reproducible 3D printing, high linearity for the Beer-Lambert relationship and biocompatibility with the glucose and lactose assay systems were considered.

3.1 Evaluating transparent 3D printer filaments for applications in UV/VIS spectroscopy

Using a design of identical dimensions to a standard disposable polystyrene cuvette, 46.0 mm x 10.8 mm x 10.8 mm (H x W x D), cuvettes of HD Glass, T-Glase and colourless PLA were 3D printed. The optical behaviour of each filament, in the printed state, was evaluated by placing the 3D printed cuvettes into a UV/VIS spectrophotometer and scanning the absorbance from 200 to 800 nm. The final 3D printed cuvette used for these studies had walls of 0.4 mm thickness, the thinnest continuous single shell wall printable on the Makerbot Mini 3D printer. In principle, the thinner the walls used to construct the devices the greater the light transmission through the 3D print. This is partly due to the Beer-Lambert law, however since FFF 3D printers construct walls from multiple extruded layers, thicker walls equate to more extruded layers, more defects, more lensing and greater light scattering. Figure S1 shows how light transmission through the filament varies with 3D print wall thickness and confirms that maximum light transmission occurs with a 0.4 mm wall.

The vertical height of the extruded plastic layers in FFF printing also impacts the amount of optical scattering in 3D prints [20], so test cuvettes were printed at several different layer heights, comparing their optical behaviour to disposable polystyrene cuvettes. Figure 1 shows the optical properties of each of the filaments when used to construct 3D printed cuvettes at 0.1 mm, 0.2 mm and 0.3 mm layer heights, both for the native material (uncoated) and coated internally and externally with XTC-3D®.

Figure 1 3D Printer filaments evaluated for applications in UV/VIS spectroscopy. Red circles show the absorption behaviour of disposable polystyrene cuvettes. Filled blue diamonds, filled black triangles and green crosses show data for 3D printed cuvettes with 0.1 mm, 0.2 mm and 0.3 mm layer heights, respectively. (1A) The absorption spectrum of native HD Glass and (1B) HD Glass coated with XTC-3D®. (1C) the

absorption spectrum of native T-Glase and (1D) T-Glase coated with XTC-3D[®]. (1E) the absorption spectrum of native colourless PLA and (1F) colourless PLA coated with XTC-3D[®]. Data are the mean average absorbance of three different 3D prints $\pm$ SD.

Figure 1 suggests that all three filaments could be used to construct 3D printed devices with applications in UV/VIS spectroscopy. In the native printed state all materials have a working range of 350 to 800 nm which is comparable to the working range of polystyrene cuvettes. When compared to polystyrene cuvettes, light transmission through the 3D printed cuvettes is much less with a typical apparent absorbance of 1 to 2 observed. This is almost certainly optical scatter, due to the lensing effects of the individual 3D printed layers. In general, the standard deviation in apparent absorbance is greater for the 3D printed cuvettes when compared to the polystyrene cuvettes. There is some variation in this error with print layer height, which is probably the result of optical scatter and small layer defects in the print.

Figure 1 shows that post-print treatment with XTC-3D[®] significantly increases light transmission for 3D printed HD Glass and T-Glase cuvettes. In the native state these materials have an apparent absorbance in the range of 1 to 2 a.u. and this value reduces to 0.2 to 0.4 a.u. after the post-print treatment. For PLA there is also a reduction in apparent absorbance after post-print treatment, however this reduction is not as significant as that seen for HD Glass and T-Glase. Figures 1E and 1F suggest the combination of PLA and XTC-3D[®] is unlikely to be useful for highly sensitive UV/VIS applications. The refractive indices of PLA, HD Glass and T-Glase are 1.465 [37], 1.522 [38], 1.575 [37], respectively. XTC-3D[®] is an epoxy resin and epoxy resins have refractive indices in the range of 1.50 to 1.56 [39]. Therefore, it is likely that the better performance of XTC-3D[®] with HD Glass and T-Glase is due to the similar refractive indices of these materials. This is consistent with the post-print treatment filling voids between extruded layers which reduces lensing effects and light scatter.

Changing the individual layer heights of the printed filaments influences light transmission for all materials studied, Figure 1A shows this most clearly for HD Glass in the native printed state. When coated with XTC-3D[®], the effect of layer height on light transmission is less significant, see Figure 1B for an example, which is consistent with the epoxy resin filling gaps between the layers and reducing light scattering. The best results, those printed systems with the lowest apparent absorbances and highest light transmission, were given by HD Glass and T-Glase printed at layer heights of 0.1 mm and 0.3 mm, respectively. XTC-3D[®] improves light transmission for all the filaments but it also decreases the working wavelength range of 3D prints due to an absorbance occurring at around 400 nm. Another disadvantage of using XTC-3D[®], at least in the interior of 3D prints, is that it may introduce chemical compatibility issues that are not present in the native plastic. However, an advantage of XTC-3D[®] is that it improves the water-tightness of the final 3D prints as no leakage was experienced after application. Although, it should be noted that print fail rates due to leaking on uncoated prints were already very low (about 1 in 10) using the Makerbot Mini 3D printer and the reported 3D print settings.

Overall the data in Figure 1 suggest that HD Glass and T-Glase are better suited than PLA to construct 3D printed devices for applications in UV/VIS spectroscopy. As PLA has a glass transition temperature of $57.3 \pm 0.5^{\circ}\text{C}$, compared to HD Glass ($75.8 \pm 0.7^{\circ}\text{C}$) and T-Glase ($77.7 \pm 0.7^{\circ}\text{C}$) [26], 3D printed devices constructed from HD Glass and T-Glase also have a higher working temperature than devices constructed from PLA.

3.1 A chemical assay of glucose performed in 3D printed cuvettes

To further characterise these filaments for UV/VIS applications, a colorimetric chemical assay for glucose [36] was performed in PLA, HD Glass and T-Glase cuvettes. Layer heights that gave the highest light transmission in Figure 1 were used for these studies, i.e. PLA (0.1 mm), HD Glass (0.1 mm) and T-Glase (0.3 mm). This experiment was designed to test if good adherence to the Beer-Lambert law could be obtained over

a range of concentrations compared to a parallel assay performed in disposable polystyrene cuvettes.

Figure 2 Evaluation of 3D printed cuvettes in a colorimetric glucose assay. (2A) Cuvette design, (2B) final 3D printed cuvette coated with XTC-3D®; responses of cuvettes to glucose (2C) in standard polystyrene cuvettes ($R^2 = 0.999$), (2D) PLA, 0.1 mm layer height ($R^2 = 0.9945$), (2E) T-Glase, 0.3 mm layer height ($R^2 = 0.9965$), (2F) HD Glass 0.1 mm layer height ($R^2 = 0.9976$). Data are the mean average absorbance of three repeats $\pm$ SD.

Figure 2C shows the glucose assay performed in polystyrene cuvettes, in general, error bars demonstrate that assay system used produces reliable results. Unless flushed with nitrogen gas, the developers of the assay report [36] that this particular glucose assay is non-linear below 1mg/ ml, due to contaminating oxidation products. Figure 2C shows similar behaviour to the literature [36] and is non-linear below 0.1 mg/ ml glucose.

Figures 2D, 2E and 2F show data for PLA, HD Glass and T-Glase, respectively. All

have a similar gradient to Figure 2C and lose linearity below 0.1 mg/ ml glucose. The error bars indicate that the assays performed in these cuvettes are reproducible, however the error bars are greater than those produced by the polystyrene cuvettes. The errors increase as the magnitude of the absorbance increases for all 3D printed cuvettes, particularly PLA (Figure 2D) probably because the upper limits of sensitivity of the UV/VIS spectrometer are being reached.

Overall the data in Figures 1 and 2 suggest all filaments coated with XTC-3D® could be used for 3D printed UV/VIS spectrophotometric applications. Linearity is observed up to an absorbance of at least 2.5 a.u. and, although optical transmission through these materials is less than for commercially available polystyrene cuvettes, the transmitted light is of sufficient intensity to give an accurate linear response.

3.2 3D Printed, UV/VIS compatible, enzyme-based detection systems: filament compatibility with enzymatic assays of lactose and glucose

To assess the design criteria of incorporating multiple enzyme catalysed reactions within a single 3D printed device, coupled enzyme assays for lactose and glucose were used. Before implementing these assays within 3D printed devices, biocompatibility tests were performed by 3D printing small disks that were immersed in the assay mixtures. These tests used printed filaments (rather than sections cut from the spool) because the heating temperature required to extrude the filaments (ca. 230°C) can cause degradation products that may impair the enzyme assay. Figure S3 shows the results of these studies, which indicate that both assays could be performed in the presence of PLA, HD Glass and T-Glase filaments. In some instances, notably HD Glass, there was a small reduction in absorbance at the assay endpoint in the presence of the filament, compared to the control. It is not clear if this was due to enzyme inhibition by the filament, see Figure S3, or an interaction between the filament and the reagents, that reduces the amount of free substrate in solution. Most importantly, these data demonstrate that the lactose/ glucose assays are compatible with PLA, HD Glass and T-Glase filaments.

3.3 3D Printed, UV/VIS compatible, enzyme-based detection systems: enzyme immobilisation in 3D printed devices

Coupled enzyme assays are typically performed in a single reaction chamber. However, to explore the flexibility that FFF offers, coupled assays were performed in a single reaction chamber and deconstructed so that the different enzymatic steps took place in up to three different compartments of the 3D printed device. In these designs, liquids are transferred between compartments by tipping the device in different directions. However, to be successfully implemented, each of the enzymes in the assay had to be immobilised. There are several advantages to immobilising the enzymes in separate compartments, the first being that decanting the reactants and products away from the enzymes stops the reaction. The second is that the same bioanalytical devices can be used to perform several different assays simply by placing the sample in a different starting chamber. Finally, deconstruction of the assay pathway can make it easier to identify the site of action of pathway inhibitors in some assays.

Several different methods of enzyme immobilisation to 3D prints were evaluated. The first was non-specific absorption of the proteins directly to the 3D print. Secondly, enzymes were conjugated to gold nanoparticles and attempts were made to immobilise these on the print through non-specific binding or by application within a solvent matrix, direct to the 3D print. Neither of these methods was effective at immobilising active enzymes [17], therefore, enzymes were conjugated to magnetic beads (Dynabeads™) and small neodymium magnets were embedded within the 3D prints to hold the enzyme in place. Figure S2 shows the steps taken to insert the magnets into the 3D print.

Figure 3 shows the design of a 3D printed device containing three reaction chambers. Figures S4 and S5 show different variations of the design with one or two reaction chambers. Key features of the design are an analysis chamber and separate reaction chambers (Figure 3A and 3B). Neodymium magnets were inserted into the 3D print midway through the 3D printing process, (Figure 3C). Prior to the addition of the assay reagents proteins immobilised on magnetic beads were added to each of the

chambers 1 (β -Gal), 2 (GOD) and 3 (HRP). Assay reagents were added to chamber 1, 2 or 3 (Figure 3C), depending on the assay requirements and solutions were transferred between reaction chambers by rotating the device. After the final reaction step the reaction mixture was decanted into the analysis chamber by a further rotation of the device. Figure 3D shows one of the devices filled with blue liquid to highlight the lower analysis chamber.

Figure 3 Design details for a 3D printed bioanalytical device with three reaction chambers. (3A) Front left, (3B) left side, (3C) front right (top surface removed for clarity), (3D) device with solution in the analysis chamber.

To confirm that the enzymes remained stably bound to the bead and the bead was held in place by the magnet for the duration of the assay a series of control experiments were performed (Figure S6). In these control studies, solutions of the assay reagents, minus the substrates, were incubated with enzymes bound to the

beads and held in place by the magnets. After decanting from the bound enzymes, the assay substrates were added and incubated for the standard assay duration. These control studies showed identical absorbance to blank control studies, where no substrate had been added, indicating that no enzyme was present in the supernatant solutions.

3.4 3D Printed, UV/VIS compatible, enzyme-based detection systems: multiple enzyme catalysed reactions performed and analysed in a single 3D printed device

The findings in 3.1 to 3.2 show that PLA, HD Glass and T-Glase coated with XTC-3D® are suitable for UV/VIS compatible 3D printing applications. HD Glass was selected to construct the final bioanalytical devices because fewer 3D print failures were experienced for this material when tested on several different 3D printers. The efficacy of the 3D printed bioanalytical devices was tested using a coupled lactose assay within a single compartment, a deconstructed coupled assay system using a two-step glucose assay (two compartments) and a three-step lactose assay (three compartments). For rigor, assays performed in these 3D printed systems were compared to the same assays performed in polystyrene cuvettes, using both Amplex Red and o-dianisidine in the different 3D printed systems. Each of these studies should obey the Beer-Lambert law, if the 3D printed devices are reproducible and effective across the range of substrate concentrations. Figure 4 shows a summary of results.

Figure 4 Spectrophotometric behaviour of transparent 3D printed devices containing one, two or three reaction chambers. (4A) Transmission of light through the empty device, constructed from HD Glass, in the native printed state (black dots) and with a single exterior coat of XTC-3D® (grey dots). (4B) Final absorbances of a two-step glucose assay, performed in separate reaction chambers, versus starting glucose concentration (unfilled squares, $R^2 = 0.9686$) and a control study (filled black circles, $R^2 = 0.9955$) performed as a standard coupled assay in a polystyrene cuvette. (4C) Final

absorbances of a three-step lactose assay, performed in a single reaction chamber, versus starting lactose concentration (unfilled squares, $R^2 = 0.9960$) and a control study (filled black circles, $R^2 = 0.9942$) performed as a standard coupled assay in a polystyrene cuvette. (4D) Final absorbances of a three-step lactose assay carried across three reaction compartments versus starting lactose concentration (unfilled squares, $R^2 = 0.9965$) and a control study (filled black circles, $R^2 = 0.9984$) performed as a standard coupled assay in a polystyrene cuvette. Data are the mean average of three repeats $\pm$ SD.

Figure 4A shows that the device printed in HD Glass is optically transparent and that a single exterior coat of XTC-3D[®] caused a significant increase in light transmission at all wavelengths studied. As observed with the simpler 3D printed cuvette design, XTC-3D[®] reduced the shortest working wavelength to around 400 nm. When a coupled glucose assay in a two-step 3D printed device was performed using o-dianisidine as the colorimetric sensor (Figure 4B) and in a polystyrene cuvette good linearity ($R^2 = 0.99$ and 0.96) was achieved, although the error bars were larger in the 3D printed system, probably due to print variability. The difference in the gradient between the control study (blue line) in Figure 4B and the assay in the 3D printed device is probably due to the inhibitory effect of HD Glass in the enzyme assays (Figure S3). Although, the way that the assay is constructed, i.e. through deconstructed steps (3D printed device) or in a single pot (polystyrene cuvette, control), might also cause the observed reduction in absorbance.

Figure 4C shows the lactose assay performed with the three different proteins separately immobilised in a single compartment and Amplex Red as the indicator. There is excellent linearity in the assay and once again the gradient is slightly less than in the control study, performed in polystyrene cuvettes. Figure 4D shows the lactose assay deconstructed across 3 compartments with Amplex Red as the indicator alongside the polystyrene cuvette control. Again, there is excellent linearity and a lower gradient compared to the control, which is probably due to the inhibitory effects of HD Glass (Figure S3). Figure S7 shows the lactose assay performed at 20°C and 37°C, for

comparison. There is good linearity at both temperatures demonstrating that this 3D printed bioanalytical system can be used at room temperature, potentially making it more widely usable. Broadly speaking, the sensitivity of the 3D printed assay systems is comparable to the same assays performed in polystyrene cuvettes. Testing versions of the device that contain one, two or three different reaction chambers demonstrates that the design principles employed are versatile and robust.

4.0 Discussion

A number of previous studies have used FFF for spectroscopic applications and it is beneficial to consider how the work in the current study fits with the context of this prior literature. Typically, in most published studies the optically transparent part of the device is not 3D printed. For example, Pisaruka et al. [14] used FFF to construct a water-heated cuvette but quartz slides were glued on to provide optically transparent windows for the analysis chamber. Similarly, for UV/VIS flow chemistry applications, polystyrene slides were used by Michalec et al. [25], whilst, Michalec and Tymecki [23] 3D printed cuvette inserts for flow chemistry applications using commercial plastic cuvettes to obtain good spectroscopic data. Vaněčková et al. [22] utilised quartz cuvettes to enable optical detection in their 3D printed spectroelectrochemistry devices. A similar approach utilising plastic cuvettes was used by Brisendine et al. [18] and Grasse et al. [19]. Herein, transparent filaments were used to construct the entire 3D printed device, which is similar to the approach taken by Tothill et al. [20] to construct 3D printed microfluidic devices analysable with UV/VIS spectroscopy. Similarly Roda et al. [24] utilised transparent ABS produced by FFF for smartphone detection systems.

Another consideration for future applications is filament biocompatibility, since a significant advantage of FFF printing is that a large range of different materials can be used for fabrication. Several studies [16,25,40] have performed chemical compatibility studies on outlook of the chemical compatibility of the PLA, T-Glase and HD Glass filaments assessing how either the FFF print mass/ dimensions or the filament mass/ dimensions change after immersion in different chemicals. In summary, PLA filament

mass shows only a small change in aqueous solutions at neutral and acidic pH, although alkaline aqueous solutions cause filament breakdown [16]. PLA also showed good compatibility with ethanol, which is consistent with many studies that show the PLA polymer has good stability in alcohols and poor stability (high miscibility) with chlorinated solvents [41,42]. Recent work by Heikkinen et al. [40] compares the chemical compatibility of a range of FFF materials, including PLA, HD Glass (polyethylene terephthalate glycol) and T-Glase (polyethylene terephthalate). All three materials show good stability in water and alcohols, but less stability when immersed for 7 days in concentrated acids and alkalis. This future direction of this work is biosensing applications adapting the present system to incorporate lipids [43], membrane interacting proteins [33] or transcription/ translation assays [44,46]. The future applications envisaged require aqueous chemical systems at approximately neutral pH to ensure the stability of the components used (enzymes, DNA, lipids). Consequently, there are unlikely to be any chemical compatibility issues with these filaments for this type of application.

5.0 Conclusions

In this study a series of FFF 3D printed devices, constructed from commercially available materials, were presented. The final devices were optically transparent, have a working range of wavelengths from 350 to 800 nm and were constructed as a single 3D printed part. Furthermore, three transparent filaments were characterised for potential applications in UV/VIS spectroscopy and it was demonstrated that coating 3D prints with XTC-3D® increases their optical transparency. Addition of XTC-3D® also decreases leaking in the final 3D prints, which makes it a very useful post-print treatment for fluid-handling 3D prints. It should be noted that using the Makerbot Mini 3D printer for this work very few leaks were experienced, even without post-print treatment with XTC-3D®. On the other hand, different commercially available FFF 3D printers provide the end user with a different level of control over the default print settings, so print failure due to leaking can vary for identical designs printed on different

FFF printers. This study suggests that coating 3D prints with XTC-3D® is a time efficient way to prevent print leakage, assuming there are no chemical compatibility issues.

Enzymes were immobilised in FFF 3D prints by coupling them to magnetic beads, held in place by magnets embedded inside the 3D print. Immobilised enzymes retained their activity and remained bound to the 3D print throughout the assay. A series of transparent bioanalytical devices were constructed using HD Glass. The 3D printed system presented is robust, versatile and easily adapted to different numbers of coupled enzymes. It contains separate reaction and analysis chambers and liquids are easily moved around the device by rotation. All designs fit into a standard UV/VIS spectrophotometer through the cuvette holder and assays performed in them show comparable linearity and sensitivity to the same assays performed in standard polystyrene cuvettes. Overall this work demonstrates that a combination FFF and transparent filaments can be used to construct sensitive and versatile tools for biosensor development.

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Competing Interests

The authors declare no competing interests.

Appendix A: Supplementary data

Supplementary data to this article can be found online at

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