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Direct 3D printing of monolithic ion exchange adsorbers

Ursula Simon & Simone Dimartino*

Institute for Bioengineering, The School of Engineering, The University of Edinburgh,
Edinburgh EH9 3DW, UK.

*Corresponding author: Phone: +44 131 6507305, Email: simone.dimartino@ed.ac.uk

Abstract

Monolithic adsorbers with anion exchange (AEX) properties have been 3D printed in an easy one-step process, i.e. not requiring post-functionalization to introduce the AEX ligands. The adsorber, 3D printed using a commercial digital light processing (DLP) printer, was obtained by copolymerisation of a bifunctional monomer bearing a positively charged quaternary amine as well as an acrylate group, with the biocompatible crosslinker polyethylene glycol diacrylate (PEGDA). To increase the surface area, polyethylene glycol was introduced into the material formulation as pore forming agent. The influence of photoinitiator (Omnirad 819) and photoabsorber (Reactive Orange 16, RO16) concentration was investigated in order to optimize printing resolution, allowing to reliably 3D print features as small as 200 μm and of highly complex Schoen gyroids. Protein binding was measured on AEX adsorbers with a range of ligand densities (0.00, 2.03, 2.60 and 3.18 mmol/mL) using bovine serum albumin (BSA) and c-phycocyanin (CPC) as model proteins. The highest equilibrium binding capacity was found for the material presenting the lowest ligand density analysed (2.03 mmol/mL), adsorbing 73.7 ± 5.9 mg/mL and 38.0 ± 2.2 mg/mL of BSA and CPC, respectively. This novel 3D printed material displayed binding capacities in par or even higher than commercially available chromatographic resins. We expect that the herein presented approach of using bifunctional monomers, bearing commonly used chromatography ligands, will help overcome the material limitations currently refraining 3D printing applications in separation sciences.

Keywords: Additive manufacturing; Digital light processing; 3D print materials;
Anion exchange chromatography; Protein adsorption

1. Introduction

3D printing, also known as additive manufacturing (AM), encompasses a range of techniques to fabricate three dimensional objects from computer aided design (CAD) models through layer by layer addition of material. Several 3D printing methods have been developed and optimised over the last couple of decades, each with its own specifications in terms of costs, resolution, build size, speed and materials [1]. AM techniques are developing at a fast pace, and today it is possible to print complex structures with high fidelity at micron scale resolution in a rapid and robust process [1]. In addition, the cost of 3D printers is dropping significantly with time, triggering the adoption of AM methods in a range of scientific fields such as chemistry [2], drug delivery [3], microfluidic [4,5] and tissue engineering [6]. Interestingly, 3D printing is not widely employed in the separation sciences yet, especially in chromatographic separations [7]. A range of serious barriers has so far limited the use of 3D printing methods in chromatography, e.g. the lack of materials compatible with both 3D printing processing and chromatographic operations, as well as the relatively poor resolution of current 3D printers to generate features in the micron scale [8].

The performance of chromatographic separations depends on a number of factors, including the flow of the mobile phase within the column and the related axial dispersion and band broadening effects. Traditional stationary phases consist either of random beds of spherical particles or random monolithic networks. Accordingly, each column has a slightly different internal morphology and porous structure, their chromatographic behaviour is impossible to be predicted *a priori*, and they require careful testing and validation of the packing quality prior to use. Computer simulations demonstrated that ordered beds provide significantly improved chromatographic performance over randomly organised stationary phases [9,10]. In an attempt to experimentally prove the simulated results, Fee *et al.* [11] 3D printed chromatography beds with precisely controlled and highly ordered morphology. These were printed with high fidelity and reproducibility to the original CAD designs. 3D printing also opened the opportunity to explore alternative bed configurations and particle shapes, allowing experimental demonstration that spherical particles are not necessarily the best shape for chromatographic operations, and that new bed morphologies can improve pressure drop and plate height characteristics [12]. Yet, only commercially available urethane-based materials for 3D polyjet printing could be tested, and flow performance of the columns was determined by residence time distribution experiments with non-retained tracers. No separation could be carried out as the material did not present suitable functional groups, while its proprietary composition limited opportunity for targeted activation chemistry and functionalization with chromatographic ligands.

Lack of suitable materials for chromatographic separations that can be processed by 3D printers represents the current biggest hurdle for the 3D printing of separation devices. Although, there is a large range of 3D printable materials available on the market (metals, ceramics, polymers), none of it was developed to bear specific functional groups for separations. Additionally, most material formulations are proprietary, and include a range of components such as plasticizers, fillers and additives to enable their 3D printing. This greatly

limits any attempt in their functionalization and renders impossible the prediction of their potential separation behaviour.

To overcome this issue, three main approaches have been applied to 3D print separation devices. The simplest approach employs commercial materials which show some degree of separation properties. For example, MacDonald *et al.* exploited the overall negative surface charge of the Veroclear material (from Stratasys) to fabricate thin layer chromatography (TLC) platforms for protein separations [13]. Su *et al.* employed another polyacrylate based material (BV-001, Rays Optics Inc.) with electron donor groups on its surface for the fabrication of a device able to selectively extract trace elements from seawater [14]. Although this method is relatively straightforward, the lack of knowledge of the material chemistry heavily limits its extension to other separation methods e.g. relying on hydrophobic, multimodal or affinity interactions.

A second approach involves the printing of materials with known composition and chemistry, which are functionalised with appropriate chromatographic ligands post printing. This strategy was employed by Fee *et al.* to produce agarose and cellulose based stationary phase which were later functionalized with a range of chromatographic ligands [15]. Seo *et al.* printed micropatterned AEX membranes by printing the desired membrane shape with non-functionalised material which was then followed by a quaternisation procedure [16]. Such approach is at all similar to current production of chromatographic resins. Albeit it is well established and robust, it requires additional manufacturing steps and adds complexity in the processing line. Functionality can also be achieved by coating 3D printed structures using, for example, initiated chemical vapour deposition [17] or porous metal frameworks [18].

The third approach aims at custom design and 3D print the column casing only, which is later packed with commercial adsorber particles [19] or monolithic structures [20]. This approach is particularly original and can lead to column geometries with improved performance [21], but cannot be considered 3D printing of chromatography stationary phases.

All these approaches require additional steps than just 3D printing, e.g. material characterisation, post printing functionalisation or the assembly of different parts. On the other hand, direct 3D printing of chromatographic adsorbers, i.e. already containing the desired functional groups, would enable a convenient one-step fabrication method for printing separation devices.

3D printing of functional materials has been recently proven in the field of chemical catalysis, where catalytically active structures with carboxylic acid, amine and copper carboxylate functionality were directly printed using bifunctional monomers as building blocks [22]. These bifunctional monomers provided one functional group with the desired catalytic chemistry as well as one to take part in the polymerization reaction. Experiments showed that the performances of the 3D printed catalytic structures were comparable with those of commercial material.

The use of bifunctional monomers has been explored in IEX monoliths by Milton Lee's group. Fully functional porous monoliths were prepared in a single step synthesis via UV initiated polymerisation by copolymerising the crosslinker PEGDA with bifunctional monomers containing sulfonic acid [23,24], phosphoric acid [25], carboxylic acid [26] or amine groups [27]. Porosities were created by the solvents present in the reacting mixture. All produced monoliths showed good protein uptake comparable to commercial columns.

In this work, direct 3D printing was employed to fabricate chromatography stationary phases. The material developed contained AEX moieties, and its formulation was tuned and optimised for their 3D printing in a digital light processing (DLP) 3D printer. Free radical polymerisation between a PEGDA crosslinker and a bifunctional monomer bearing quaternary amine groups was achieved using Omnirad 819 as photoinitiator. Addition of a light absorber was key to tune and control the resolution of the 3D printed models, enabling reliable fabrication of complex structures with 200 μm thick features. The developed AEX material showed excellent protein adsorption behaviour for two model proteins, bovine serum albumin (BSA) and C-Phycocyanin (CPC). Optimal ligand density for protein adsorption was determined by adjusting the composition of the parent formulation before 3D printing. To the best of our knowledge, this work is the first proposing the one-step manufacture of functional stationary phases with perfectly ordered internal morphology.

2. Experimental

2.1. Materials

2-(Acryloyloxy)ethyl trimethylammonium chloride (AETAC, 80 wt. % in water), ethanol (absolute, for HPLC, $\geq 99.8\%$), hexamethyldisilazane (HMDS), Reactive Orange 16 (RO16), sodium hydroxide and Sudan I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (protease free powder), isopropanol (IPA, extra pure), polyethylene glycol (PEG-200, average MW 200 g/mol), sodium chloride, sodium phosphate mono and dibasic were obtained from Fisher Scientific (Hampton, NH, USA). Phenyl bis(2,4,6-trimethylbenzoyl)-phosphine oxide (Omnirad 819, former Irgacure 819) was kindly donated by IGM resins (Waalwijk, The Netherlands). Polyethylene glycol diacrylate (PEGDA, SR259, average MW 200 g/mol) was donated from Arkema-Sartomer (Colombes, France). C-Phycocyanin (CPC) was extracted from *Spirulina platensis* and gently provided by Dr. Alistair McCormick (The University of Edinburgh, Institute of Molecular Plant Sciences). All chemicals were used as received; all buffers were prepared using deionised water (EuRO10 Reverse Osmosis System, Evoqua Water Technologies, Pittsburgh, PA, USA).

2.2. Material development

The photocurable material was composed of 60 wt. % PEG-200 as porogen and 40 wt. % PEGDA/AETAC mixture as crosslinker and bifunctional monomer, respectively. Printed samples were created with different ligand densities by adjusting the relative ratio of PEGDA and AETAC (Table 1).

A radical photoinitiator (Omnirad 819, at 0.12, 0.25, 0.5, 1.0 and 1.5 wt. % based on 100 g of porogen-monomers mixture) and a light absorber were added to the mixture. Three dyes

were considered as light absorbers to help increase the resolution of the 3D printed models, namely RO16, Sudan I and Tinuvin 326. A NanoDrop 2000c (Fisher Scientific, Hampton, NH, USA) was employed to measure the absorbance spectra of the photoinitiator and photoabsorbers to direct selection of the appropriate light absorber. RO16 was chosen for all further experiments due to its most suitable absorbance spectrum (see Results and Discussion section). Three different concentrations of RO16 (0.063, 0.125, 0.250 wt. %, based on 100 g of porogen-monomers mixture) were tested in combination with 0.5 wt. % Omnirad 819. A final concentration of 0.125 wt. % RO16 was chosen to fabricate the prints (100 μm cure depth at 1.7 s exposure). All material formulations were stored in tubes covered in aluminium foil to prevent spontaneous polymerisation prior to 3D printing.

[Table 1]

2.3. Model creation and 3D printing

Computer-aided design (CAD) models were created on SolidWorks 2015 (Dassault Systèmes SOLIDWORKS Corp., Waltham, MA, USA), exported as STL file and sliced into 2D-layers using Netfabb 2017 (Autodesk, San Rafael, CA, USA). A unit cell of the Schoen Gyroid was created using Mathematica 10.4 (Wolfram Research Inc., Champaign, IL, USA).

A Solfex 350 (W2P Engineering, Vienna, Austria) digital light processing (DLP) printer (50 μm pixel size in x-y plane, UV-LED at 385 nm with light intensity of 16 mW/cm²) was used as 3D printing platform. All parts were printed in 100 μm layers (z-direction) as a reasonable compromise between printing resolution, column size and overall printing time. Post-printing, the parts were washed three times in IPA in an ultrasonic bath (Allendale Ultrasonics, Hodesdon, UK) and then fully cured in water with a xenon Otofash G171 unit (NK-Optik, Baierbrunn, Germany). Parts were stored in water until final use.

To determine the working curve (layer thickness vs exposure time) of the new material formulations, 8 mL of the material were transferred into a petri dish ($\varnothing$ 60 mm) and placed above the printing area of the DLP. Eleven circles ($\varnothing$ 8 mm) were cured at different locations with exposure times from 1 to 60 s. After cleaning with IPA and post-curing, the thickness of the polymerised circles was measured using a micrometre (RS Pro Micrometer External, RS Components, Corby, UK).

2.4. Qualitative characterisation of porous network

The internal structure of printed parts was imaged through scanning electron microscopy (SEM, S-4700, Hitachi, Tokyo, Japan). Cylindrical gyroidal structures (50 % external porosity, 500 μm wall thickness) were printed and then dehydrated in 70 %, 80 %, 90 % and 100 % ethanol, with each step performed three times for 10 min. The gyroids were then transferred to HMDS for 2 min, HMDS was then removed and samples were left to dry [28,29]. Subsequently, the cylinders were immersed in liquid nitrogen and snapped into halves, gold sputter coated and SEM imaged.

The interconnectivity of the porous structure was tested by incubating cylindrical gyroid structures (50% external porosity, 500 μm wall thickness) with 1 mg/mL blue CPC in phosphate buffer (25 mM, pH 7) for 24 h. After incubation, gyroids were cut into halves and the cross section of the walls was visually investigated in regard of colour.

2.5. Protein batch adsorption

Hollow cylinders fitting into 96-microplate wells were designed and 3D printed (Figure 1). Prior to protein adsorption tests, printed cylinders were equilibrated with phosphate buffer (25 mM, pH 7) for a minimum of 48 h, with buffer exchange every 12 h. Protein adsorption was triggered by addition of 170 μL phosphate buffer (25 mM, pH 7) containing 0-32 mg/mL BSA or 0-8 mg/mL CPC to each well. The microplates were agitated at 800 rpm using a Thermomixer C (Eppendorf, Hamburg, Germany). Protein adsorption kinetics onto the 3D printed cylinders was calculated by protein uptake in the bulk solution, measuring protein absorbance with a Modulus II Microplate Multimode Reader (Turner BioSystems, Sunnyvale, CA, USA) at regular time intervals. The binding capacity, q , was calculated for each time point using equation (1):

$$q = \frac{(c_i - c) \cdot V}{V_{cylinder}} \quad (1)$$

where c_i and c are the initial protein concentration and the concentration at time t , respectively, V is the volume of buffer in the wells and $V_{cylinder}$ is the printed cylinder volume (19.37 μL).

The Langmuir model was employed to describe protein loading at equilibrium conditions (q_{eq}) as function of the equilibrium protein concentration in the liquid phase (c_{eq}):

$$q_{eq} = \frac{q_{max} \cdot c_{eq}}{K_D + c_{eq}} \quad (2)$$

The maximum binding capacity, q_{max} , and the Langmuir equilibrium coefficient, K_D , were estimated by best fit regression using Origin 2016 (OriginLab, Northampton, MA, USA).

BSA concentration was measured through UV readings at 280 nm, while CPC concentration was calculated from the absorbance at 615 and 652 nm according to the expression reported in [30,31], adapted for NanoDrop measurements by adding a correction factor of 0.1 to account for the shorter path length:

$$c_{CPC} = \frac{A_{615} - 0.474 \cdot A_{652}}{5.34 \cdot 0.1} \quad 3$$

The absorbance ratio of A_{615}/A_{280} is generally used to describe the purity of CPC. The absorbance at 615 nm corresponds to the maximum absorbance of CPC, whereas the absorbance at 280 nm correlates to contamination with other proteins. A ratio of 0.7 is considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade [32].

The CPC applied in experiments had a purity of $A_{615}/A_{280} = 2.3$. All batch adsorption experiments were performed in triplicate.

[Figure 1]

3. Results & Discussion

3.1. Material development

The aim of this study was the one-step 3D printing of chromatographic beds with AEX functionality. This was achieved by UV copolymerisation of the crosslinker PEGDA and the bifunctional monomer AETAC bearing positive charged quaternary amine groups (Figure 2). As second functional group, AETAC displays an acrylate group for the incorporation in the polymeric network. PEGDA was chosen as crosslinker due to its known biocompatibility and low non-specific protein binding [33]. This principle of copolymerisation has previously been reported for the fabrication of ion exchange monoliths [23–27]. In this study, 40 wt. % of the crosslinker-monomer blend was mixed with 60 wt. % PEG-200 as pore forming agent.

[Figure 2]

3.1.1. Optimization of photoinitiator and photoabsorber concentration

The concentration of the photoinitiator in the material formulation determines the thickness of 3D printed layers, commonly known as the cure depth C_D . Different concentrations of the Omnirad 819 photoinitiator were tested and the resulting C_D for an exposure time of 1 and 2 s is summarised in Figure 3a. An exposure time of 1 to 2 s was found to be desirable to ensure an acceptable printing time for monolithic structures in later experiments. At fixed exposure time, the cure depth decreased with increasing photoinitiator concentration. For instance, at 1 s exposure (i.e. a reasonably small exposure time to enable fast printing), C_D was $1456 \pm 271 \mu\text{m}$ when 0.125 wt. % Omnirad 819 was employed, whereas $408 \pm 12 \mu\text{m}$ cured layer was obtained with an eight times higher photoinitiator concentration (1.5 wt. %). At higher photoinitiator concentrations, the supplied light is entirely absorbed in a thinner layer immediately above the print surface, hence limiting light penetration into the photocurable material. Yet, the thinnest printed layer achieved at the highest photoinitiator concentration of 1.5 wt. % (limited by the solubility of Omnirad 819 in the monomers/porogen mixture) was $408 \pm 12 \mu\text{m}$ for an exposure time of 1 s. Such layer thickness was considered too large for appropriate resolution in the 3D printed models; in addition, formulations with high initiator concentrations are extremely sensitive to light, and start polymerizing before the 3D printing process is initiated, thus making its handling particularly delicate. On the other hand, C_D can be reduced by shortening the exposure time, however, extremely short exposure times are not reliably delivered by the light engine of the DLP printer. The required exposure time to cure a $100 \mu\text{m}$ layer was estimated equal to 64 ms or 344 ms for the materials containing 0.25 wt. % or 1.5 wt. % Omnirad 819, respectively (estimation using fitting parameters from Table S2). A reasonable compromise of 0.5 wt. % Omnirad 819 was selected to prepare all material formulations.

[Figure 3]

Light absorbers (or photoabsorbers, PA) are usually added to the formulation to increase control over the polymerization reaction and further decrease the cure depth. These components absorb part of the supplied light, thus lowering the penetration depth of the UV light within the material, hence reducing C_D . Figure 3b presents the absorbance spectra of three light absorbers considered in this work, RO16, Tinuvin 326 and Sudan I. All three PAs absorb light at 385 nm, i.e. the printer's output. Tinuvin 326 absorbed up to 410 nm, whereas the photoinitiator (Omnirad 819) absorbed up to 440 nm. Accordingly, Tinuvin 326 is unable to protect the formulation from early polymerisation in ambient light. Sudan I and RO16 showed similar absorbance spectra, overlapping completely with the Omnirad 819 spectrum in the UV-vis range, hence ensuring full protection in ambient light. For further investigations RO16 was chosen due to its lower health and safety risks.

Working curves (C_D vs. exposure time) for different RO16 concentrations in combination with 0.5 wt. % Omnirad 819 are shown in Figure 3c. As expected, use of higher concentrations of the PA resulted in thinner polymerised layers. The appropriate concentration of PA to be used is a compromise between its concentration in the formulation and the exposure time required to achieve a certain cure depth, i.e. an appropriate resolution of the printed part. On one hand, concentration should be as small as possible to limit its presence in the cured model part, which in turn could cause secondary issues such as non-specific protein binding and colour retention. On the other hand, light exposure should enable curing of a layer in a reasonable time and with reasonable resolution. As stated earlier, the target in this work was the printing of 100 μm layers in 1 - 2 s. At the smallest RO16 concentration tested (0.06 wt. %), an exposure time of 1 s resulted in a C_D of $286 \pm 31 \mu\text{m}$, i.e. a 30 % reduction with respect to the parent formulation with no PA. At 0.25 wt. % RO16, 1 s exposure did not cure a measurable layer, and approximately 2.4 s would be required to cure a 100 μm thick layer (according to logarithmic fit). An intermediate concentration of 0.125 wt. % RO16 led to a C_D of $100 \pm 30 \mu\text{m}$ in 1 s UV exposure, equivalent to a 75 % reduction with respect to the formulation with no PA. This concentration was employed in all further formulations and experiments.

3.1.2. *Printability and resolution of the new material formulation*

To investigate the printability and resolution of the material formulation here developed, a test cube was designed and 3D printed (Figure 4a and b). The cube contains square channels of 500 μm width separated by walls with thickness ranging from 200 to 1000 μm . The design of the test cube is symmetrical and such that resolution over all three printing directions (x, y and z) can be investigated at the same time, regardless of the orientation of the cube. As can be observed in Figure 4b, all walls were neatly printed, demonstrating the ability to reliably print up to 200 μm thin features, i.e. two 100 μm printed layers.

Conventional IEX adsorber beads for preparative chromatography have diameters ranging from 15 to 200 μm , with average diameter of 90 μm [34]. The structures 3D printed with the new IEX material have feature size comparable with those of commercial chromatographic resins. Yet, this study aims at presenting the proof the concept of direct printing of

functional materials for IEX. Further increase of the print resolution (50 to 100 μm range) is currently being investigated and will be the focus of future reports. The capability of 3D printing technologies to enable manufacturing of optimized three dimensional ordered structures has been recently discussed [8,10,12]. In particular, printing of complex monolithic structures with defined channel size, geometry and configuration tuned for specific separations is particularly attractive. Triply periodic minimal surfaces (TPMS) have been recently reported as geometries for chromatographic beds, with computer simulations demonstrating superior chromatographic performance in terms of permeability and axial dispersion (HETP) over random packed beds and monoliths [10]. The potential to print these complex TPMS geometries using the novel material formulation was demonstrated (Figure 4c-f). The complex Schoen Gyroid structure, designed with 50 % external porosity and 500 μm wall thickness, was reliably and accurately printed, with interconnected walls for mechanical strength and no occluded channels for fluid flow. These printed cylinders can be easily introduced into traditional chromatography columns and connected to chromatography systems (e.g. FPLC) for chromatographic separations. As printer resolution is addressed, TPMS structures with smaller features will be able to be printed and employed.

[Figure 4]

3.1.3. Qualitative study of material porosity

The formulation of the new material incorporated PEG-200 as pore forming agent. The porous network within the printed material could not be analysed by SEM as the drying operations for sample preparation caused substantial shrinkage and collapse of the porous structure. As an alternative to SEM, the porous structure and its interconnectivity was qualitatively tested by incubation of the gyroid model in CPC (Figure 4f-h), a blue protein pigment complex extracted from the cyanobacterium *Spirulina platensis*. After 24 h incubation, the original orange colour due to the RO16 dye was completely concealed by the blue characteristic of CPC (Figure 4f and g). The cylinder was cut open to demonstrate diffusion of the CPC protein within the bulk of the walls, with strong blue staining of the cut sections (Figure 4h). As a result, it was concluded that the pores within the polymerised network were, on average, larger than the diameter of the CPC protein complex (11 nm) [35] and that the pores were highly interconnected. Typical soluble proteins have a diameter of 3 - 6 nm [36], suggesting that the internal porous network is suitable for protein adsorption. Further quantitative work on the porosity characteristics of the 3D printed materials is required, both to improve the current material in terms of protein adsorption, but also to enable use of this material towards other separation targets such as DNA and viruses (VLPs, viral vectors, etc.).

3.2. Protein batch adsorption

Batch adsorption experiments were performed to analyse the protein adsorption behaviour of the novel material formulations. The advantage of 3D printing to fabricate three dimensional shapes from CAD models was employed here to simplify execution of the

experiments. In particular, the adsorbers were designed as hollow cylinders with 5 mm outer diameter and 3.5 mm height as to fit in a well of a UV transparent 96-well plate (Figure 1). The walls of the cylinders had a thickness of 500 μm to ensure sufficient mechanical properties, and presented a number of holes to facilitate mixing of the buffers and protein solutions.

In batch adsorption experiments with traditional chromatographic beads, protein concentration is measured by withdrawal of a small amount of solution followed by spectrophotometry. This method requires careful handling or a robotic platform, and inherently introduce experimental errors due to the change of the overall volume of buffer throughout the experiment. This is particularly true in kinetic essays, i.e. where protein concentration has to be monitored frequently. The hollow cylinder design here proposed, enabled by 3D printing, allowed to measure protein concentration using a simple plate reader, with no need to either remove the adsorber from the wells or withdrawal of the protein solutions, greatly simplifying execution of the experiments.

The effect of ligand density on protein adsorption was preliminary tested using BSA as model protein. Four different material compositions, corresponding to ligand densities of quaternary amine functionality of 0.00 (control), 2.03, 2.60 and 3.18 mmol/mL, were prepared and printed in the hollow cylinder format. BSA is considerably smaller than CPC [37], thus it is reasonable to assume that the material's internal porous structure is accessible to BSA. Figure 5a presents the BSA binding kinetics onto the AEX material with a ligand density of 2.03 mmol/mL (kinetics for the materials with 2.60 and 3.18 mmol/mL ligand density can be found in Fig. S2). The adsorption kinetics was relatively fast, reaching equilibrium conditions after a few hours only (dependent on initial BSA concentration). In particular, at the lowest concentrations tested, equilibrium is reached after 1-3 hours, while equilibrium was reached in less than 24 h at the highest BSA concentration tested. Commercial bead based chromatography resins generally reach equilibrium within a couple of hours [31,38–40], however, their characteristic dimension for diffusion (i.e. bead size) is about 10 times shorter than the 3D printed cylinder walls (500 μm). The adsorption kinetics observed results from a combination of different mass transport mechanisms, including boundary layer mass transfer, diffusion within the internal pores, and adsorption kinetics. The relatively thick walls of the cylindrical adsorbers (500 μm) suggests that diffusional resistance might be the limiting factor for adsorption. This is particularly true at higher protein concentrations, i.e. where diffusion may be hindered by previously adsorbed protein molecules.

Equilibrium adsorption isotherms were measured using the data obtained after 24 h incubation (Figure 5b). The Langmuir model was employed to best-fit the adsorption isotherms, with model parameter summarized in Table 2. The highest binding capacity of 73.7 ± 5.9 mg/mL was achieved with ligand density of 2.03 mmol/mL. Higher ligand densities resulted in significantly lower binding capacities (57.4 ± 2.6 and 23.3 ± 2.5 mg/mL for 2.60 and 3.18 mmol/mL ligand density), indicating lower pore diffusivity and ligand accessibility as the ligand density increases. Similar trends of protein binding capacity with ligand density

are relatively common [41,42] and justify the need to determine the optimum ligand density when a new material is being developed. It is not excluded that lower ligand densities may lead to improved binding characteristics for pre-functionalized 3D printed materials. The control adsorber, i.e. 3D printed without functional monomer, showed detectable albeit low non-specific protein adsorption (less than 10% with respect to the material with 2.03 mmol/mL ligand density) in agreement with other research findings [33]. Low non-specific binding is a desirable characteristic for stationary phases, supporting the use of the herein developed material as monolithic adsorbent for chromatography.

Commercial chromatographic resins with quaternary amine functional groups show equilibrium binding capacities of over 100 mg/mL for BSA. For example, Streamline Q XL media from GE Healthcare, a resin for expanded bed adsorption chromatography, has maximum binding capacity of 170 ± 5 mg/mL and equilibrium coefficient of 8 ± 4 mg/mL for BSA [43]. Q-Sepharose FF, another AEX resin from GE Healthcare, displays a maximum binding capacity of 102.4 ± 1.6 mg/mL and an equilibrium coefficient of 0.109 ± 0.011 mg/mL [44]. Both resins bear a tenfold lower ligand density than material developed in this work [45,46]. That supports the earlier observation that lower ligand densities may lead to increased binding capacities. Other chromatographic media, such as membranes and monoliths, instead display capacities in the 25-40 mg/mL range. For instance, the Sartorius's Q membrane has a reported maximum capacity of 27.7 mg/mL [47] for a ligand density of 0.18 - 0.24 mmol/mL [48], while BIA CIM-QA monoliths are endowed with dynamic binding capacities of 30-40 mg/mL for BSA [49] and ligand densities of about 1.1 mmol/mL [50]. Li *et al.* prepared an AEX monolith using similar chemistry as described in this work and reported a dynamic binding capacity of 56 mg/mL for a ligand density of 2.63 mmol/mL [27], comparable to the material with intermediate ligand density presented in this work (note Li *et al.* determined binding capacity in dynamic conditions). The novel 3D-printable material here presented displays somewhat lower binding capacity than traditional bead based resins (approx. 0.4 times than Streamline Q XL and 0.7 times than Q-Sepharose), but significantly higher than membranes and monoliths. These results are extremely promising, considering this is the first reported attempt to develop a pre-functional 3D printable material for chromatography. Optimisation of the material's composition, ligand density and separation conditions has great potential to improve the adsorption performance of such 3D printable materials.

[Figure 5]

The best performing material with 2.03 mmol/mL ligand density was selected for further adsorption experiments with CPC. Its strong blue colour facilitated visual observation of the adsorption process with time, with apparent colour changes of both the protein solution and the adsorber cylinders. In particular, a noticeable decrease in colour of the supernatant was observed for all CPC concentrations investigated (Figure 6a). At the same time, the adsorber cylinders turned from the initial orange colour to blue (Figure 6b), with stronger

blue shade at higher CPC concentrations. This visually proved successful adsorption, with transfer of the protein from the liquid to the solid phase.

Protein adsorption was quantitatively confirmed by spectrophotometry, with equilibrium adsorption data of CPC onto the newly developed AEX material summarized in Figure 6c and best-fit Langmuir parameters in Table 2. Data for CPC adsorption onto quaternary amine resins is particularly scarce in the literature, limiting the comparison between different chromatographic materials. The only data equilibrium data for CPC adsorption available are for the Streamline Q XL [38] and Q Sepharose FF [31] (extracted from [38] and [31] and reported in Figure 6 c to ease comparison). As opposite to what observed with adsorption of BSA, the novel 3D printed AEX material displayed higher CPC adsorption capacity than the commercial AEX resins, namely 1.4 times and 1.7 higher q_{max} for Streamline Q XL and Q-Sepharose, respectively. The affinity towards the CPC protein complex is on the same order of magnitude for the three materials, with the 3D printed adsorber having slightly better affinity compared to Streamline Q-XL (halved k_D) and very similar to Q-Sepharose.

[Figure 6]

Interestingly, the adsorption capacity significantly drops from BSA to CPC, irrespective of the material considered. However, while the drop-in binding capacity from BSA to CPC was a dramatic 83 % in the Streamline Q XL and 78% in Q-Sepharose material, it only dropped of 40 % in the 3D printed AEX material. This difference is not related solely to the different properties of the two proteins such as their difference in size (67 kDa BSA [44] vs. 112 kDa CPC [51]), their isoelectric point (4.9 for BSA [44] vs. 5.8 for CPC [52]) and the distribution of charged and hydrophobic patches on the outer protein shell, but also to the material properties e.g. ligand density, size and interconnectivity of internal porous structure. This observation highlights, again, the importance of material optimization towards the specific target protein of interest. Current work is targeting material optimisation in regard of porous structure, ligand density and mechanical properties. Other than optimization of material properties, inherent matter for researchers in the material and separation sciences, 3D printing enables the tuning of the geometry for the stationary phase as well as the mobile phase, thus offering an additional factor for further process optimization.

[Table 2]

4. Conclusions

This work demonstrates, for the first time, the potential to directly 3D print fully functional stationary phases for protein separations in one simple manufacturing step. In particular, anion exchange adsorbers were fabricated from a 3D printable material formulation comprising i) a bifunctional monomer bearing quaternary amine groups as AEX ligands, ii) a crosslinker to impart mechanical stability to the polymeric network, iii) a photoinitiator to trigger the polymerization reaction upon exposure to UV light, iv) a pore forming agent to

increase the surface area of the resulting stationary phase, and v) a photoabsorber to increase the resolution of the 3D printed part. This mixture enabled 3D printing of complex structures such as the Schoen gyroid with high fidelity, as well as to fabricate parts with 200 μm large features. Even though this feature size is on the top range of commercial chromatography resins, upcoming developments in 3D printing technologies are expected to bring the resolution below the 100 μm threshold.

3D printed anion exchange structures were tested for adsorption of BSA and CPC, revealing excellent adsorption characteristics, in line or even superior than those of commercially available quaternary amine adsorbers. It is worth noting that such results were obtained after a basic optimization of the material composition and 3D printing settings. Additional optimization efforts, e.g. on the porous structure of the cured material, print resolution, and material formulation, has the potential to further improve the adsorption characteristics of such 3D printable AEX chromatographic materials. Also, use of other bifunctional monomers enables extension of this approach to fabricate columns that could operate in other chromatographic modes. For example, 2-carboxyethyl acrylate [26] and sulfopropyl methacrylate [23] could produce cation exchange materials, while butyl methacrylate and 2-hydroxyethyl methacrylate could be part of the formulation to fabricate hydrophobic interaction columns [53].

The capability of 3D printing to create complex three-dimensional models directly from CAD designs was exemplified in this work. Adsorbers were designed and 3D printed as hollow cylinders, significantly simplifying execution of batch adsorption experiments in 96 well plate format. This concept can be extrapolated in the future, with columns designed and fabricated with new internal geometries, with improved chromatographic characteristics to suit specific separation and purification applications.

We believe the results presented here enable the first steps to overcome the current limitation in terms of materials compatible with 3D printing operations and at the same time suitable for chromatographic separations. This has the potential to revolutionise the chromatography arena, with fine-tuned and robust columns locally 3D printed, on demand and over a short period of time (e.g. overnight) as opposed to the current paradigm of “one-column-fits-all-applications” produced in centralized manufacturing facilities.

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7. Tables

Table 1: Composition of the different resin formulations.

Ligand density [mmol/mL]	AETAC [wt. %]	PEGDA [wt. %]	PEG-200 [wt. %]
0	0	40	60
2.03	14	26	60
2.60	18	22	60
3.18	22	18	60

Table 2: Langmuir parameters for BSA and CPC adsorption onto the novel 3D printed material in comparison to commercial chromatography resins. Displayed errors correspond to standard error.

		3D printed material			Streamline Q XL	Q Sepharose FF	Sartorius Q membrane
		2.03 mmol/mL	2.60 mmol/mL	3.18 mmol/mL	0.23 - 0.33 mmol/mL [45] ¹	0.18- 0.24 mmol/mL [46] ¹	0.07 – 0.18 mmol/mL [48] ²
BSA	q_{max} [mg/mL]	73.7 ± 5.9	57.4 ± 2.6	23.3 ± 2.5	170 ± 5	102.4 ± 1.6 ³	27.2
	k_D [mg/mL]	0.381 ± 0.108	0.274 ± 0.048	0.128 ± 0.075	8 ± 4	0.109 ± 0.011 ³	0.054
	R ²	0.92	0.98	0.75	-	-	0.99
	Data source	This work	This work	This work	[43]	[44]	[47]
CPC	q_{max}	38.0 ± 2.2			28.1 ± 0.1	22.7	
	k_D	0.041 ± 0.010			0.082 ± 0.001	0.031	
	R ²	0.97			0.96	0.98	
	Data source	This work			[38]	[31]	

¹ Data given in mmol Cl⁻/mL, assumed same molar concentration between Cl⁻ and Q-ligand

² Data given in µeq/cm², assumed normal concentration equals to molar concentration, converted to mmol/mL using membrane thickness of 275 µm [47]

³ Data given in mmol/L, converted to mg/mL using molecular weight of BSA (67 kDa).

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Figure captions

Figure 1: Printed adsorber in form of a hollow cylinder. a) CAD model, b) photograph of printed cylinder, c) cylinders in 96-microplate wells. The hollow cylinder format allows convenient measurement of the protein concentrations using a plate reader.

Figure 2: Chemical structures of a) PEGDA and b) AETAC.

Figure 3: AEX material development. a) cure depth, C_D , as function of Omnirad 819 (photoinitiator) concentration for exposure times of 1 and 2 s. Monomer to crosslinker ratio of 14:26 (2.03 mmol/mL ligand density). b) Absorbance spectrum of Omnirad 819 (50 ppm by weight in IPA) and the analysed dyes RO16, Sudan I and Tinuvin 326 (12.5 ppm in IPA/H₂O). Printer's output wavelength was 385 nm (black dotted line). c) Working curves at different concentrations of RO16 as photoabsorber. Other components are 0.5 wt. % Omnirad 819 and monomer to crosslinker ratio of 14:26 (2.03 mmol/mL ligand density). Error bars in a) and c) correspond to standard deviation. Green dotted lines correspond to the target layer thickness of 100 μm . Fitting parameters in supplementary Table S1.

Figure 4: Testing printability, printing resolution and porous structure of the new material formulation (ligand density 2.03 mmol/mL). a) CAD model and b) photograph of test cube with 500 μm channels, separated by 200 to 1000 μm thick walls. c) CAD model and d,e) SEM images of a Schoen gyroid with a designed external porosity of 50 % and wall thickness of 500 μm . Some degree of shrinkage can be noticed as a result of drying for sample preparation. f) Photograph of printed cylindrical Schoen gyroid before and g) after 24h incubation with 1 mg/mL CPC. h) Model cut open after 24 h of incubation with 1 mg/mL CPC.

Figure 5: BSA batch adsorption experiments. a) Adsorption kinetics onto novel material formulation with ligand density of 2.03 mmol/mL. Initial BSA concentration of 0.5 – 6 mg/mL. b) Equilibrium adsorption data for the four materials tested (ligand densities of 0.00, 1.81, 2.32 and 2.84 mg/g). Continuous lines are best-fit according to Langmuir model. Fitting parameters are listed in Table 2. Error bars correspond to standard deviation.

Figure 6: CPC batch adsorption onto novel 3D printed AEX material with ligand density of 2.03 mmol/mL. CPC concentration at beginning of experiment of 0.000, 0.125, 0.250, 0.500, 1.000, 2.000, 4.00 and 6.00 mg/ml. a) 3D printed cylinders in multiwell plate soaked in CPC solutions of different concentration at 0, 3 and 24 h following incubation. b) Adsorber cylinders after 24 h incubation with CPC solutions at increasing concentration. c) Comparison of equilibrium adsorption data of the AEX printed material (this work), Streamline Q XL (from

[40]) and Q-Sepharose (from [31]). Continuous lines are best-fit according to Langmuir model. Fitting parameters are listed in Table 2. [Error bars correspond to standard deviation.](#)

Supplementary material

Direct 3D printing of monolithic ion exchange adsorbers

Ursula Simon & Simone Dimartino*

Institute for Bioengineering, The School of Engineering, The University of Edinburgh,
Edinburgh EH9 3DW, UK.

*Corresponding author: Phone: +44 131 6507305, Email: simone.dimartino@ed.ac.uk

Table S1: Fitting parameter for working curves in Figure 3c. Curves are fitted with $y=a*\ln(x)+b$. Displayed error corresponds to standard error.

RO16 concentration [wt. %]	a [μm]	b [μm]	R ²
0.000	406 $\pm$ 25	715 $\pm$ 43	0.97
0.063	318 $\pm$ 16	130 $\pm$ 43	0.98
0.125	188 $\pm$ 2	33 $\pm$ 3	0.99
0.250	97 $\pm$ 6	14 $\pm$ 17	0.98

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739 Figure S1: Working curves for different Omnirad 819 concentrations from 0.25 to 1.5 wt. %.
740 The green dotted line displays the target layer thickness of 100 μm . The higher the initiator
741 concentration the lower the C_D . Error bars correspond to standard deviation.

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746 Table S2: Fitting parameter for working curves in Figure S1. Curves are fitted with $y=a*\ln(x)+b$.
747 Displayed error represents the standard error.

Omnirad 819 concentration [wt. %]	a [μm]	b [μm]	R ²
0.25	384 ± 18	1157 ± 33	0.98
0.5	406 ± 25	715 ± 43	0.97
1.0	351 ± 12	315 ± 24	0.99
1.5	239 ± 15	355 ± 21	0.97

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Figure S2: BSA binding kinetics for the initial protein concentrations from 0.5 to 4 mg/mL BSA. Displayed errors represent standard deviation. (a) Material presenting a ligand densities of 2.60 mmol/mL. (b) Material with ligand density of 3.18 mmol/mL.