Fabrication of biomimetic networks using viscous fingering in flexographic printing

Pauline Brumm1,3,†, Anna Fritschen2,†, Lara Doß2, Edgar Dörsam1,3, and Andreas Blaeser2,4,∗

1 Technical University of Darmstadt, Department of Mechanical Engineering, Institute of Printing Science and Technology, Magdalenenstr. 2, Darmstadt, 64289, Germany
2 Technical University of Darmstadt, Department of Mechanical Engineering, BioMedical Printing Technology, Magdalenenstr. 2, Darmstadt, 64289, Germany
3 Collaborative Research Center (CRC) 1194—Interaction between Transport and Wetting Processes, Alarich-Weiss-Str. 10, Darmstadt, 64287, Germany
4 Technical University of Darmstadt, Centre for Synthetic Biology, Schnittspahnstr. 10, Darmstadt, 64287, Germany
† Pauline Brumm and Anna Fritschen contributed equally to this work.
∗ Authors to whom any correspondence should be addressed.
E-mail: fritschen@idd.tu-darmstadt.de and blaeser@idd.tu-darmstadt.de

Keywords: viscous fingering, vascular networks, flexographic printing, hydrogel, tissue engineering, organs-on-a-chip

Abstract

Mammalian tissue comprises a plethora of hierarchically organized channel networks that serve as routes for the exchange of liquids, nutrients, bio-chemical cues or electrical signals, such as blood vessels, nerve fibers, or lymphatic conduits. Despite differences in function and size, the networks exhibit a similar, highly branched morphology with dendritic extensions. Mimicking such hierarchical networks represents a milestone in the biofabrication of tissues and organs. Work to date has focused primarily on the replication of the vasculature. Despite initial progress, reproducing such structures across scales and increasing biofabrication efficiency remain a challenge. In this work, we present a new biofabrication method that takes advantage of the viscous fingering phenomenon. Using flexographic printing, highly branched, inter-connective channel structures with stochastic, biomimetic distribution and dendritic extensions can be fabricated with unprecedented efficiency. Using gelatin (5%–35%) as resolvable sacrificial material, the feasibility of the proposed method is demonstrated on the example of a vascular network. By selectively adjusting the printing velocity (0.2–1.5 m s⁻¹), the anilox roller dip volume (4.5–24 ml m⁻²) as well as the shear viscosity of the printing material used (10–900 mPas), the width of the structures produced (30–400 µm) as well as their distance (200–600 µm) can be specifically determined. In addition to the flexible morphology, the high scalability (2500–25 000 mm²) and speed (1.5 m s⁻¹) of the biofabrication process represents an important unique selling point. Printing parameters and hydrogel formulations are investigated and tuned towards a process window for controlled fabrication of channels that mimic the morphology of small blood vessels and capillaries. Subsequently, the resolvable structures were casted in a hydrogel matrix enabling bulk environments with integrated channels. The perfusability of the branched, inter-connective structures was successfully demonstrated. The fabricated networks hold great potential to enable nutrient supply in thick vascularized tissues or perfused organ-on-a-chip systems. In the future, the concept can be further optimized and expanded towards large-scale and cost-efficient biofabrication of vascular, lymphatic or neural networks for tissue engineering and regenerative medicine.

1. Introduction

To ensure the exchange of gases, substances and information, complex organisms such as the human body have a large number of hierarchically organized networks, such as blood vessels, lymphatic channels and neuronal pathways. Though serving completely different functions, and being distinct in their
anatomical as well as physiological nature, these conduits share similar wiring concepts and network patterns comprising stochastically distributed branches and dendritic cellular sprouts [1]. Detailed and high scale reproduction of these networks represents a milestone in the biofabrication of complex tissues and organs and thus, holds great potential for both regenerative medicine as well as for disease modelling or pharmaceutical and biochemical research [2, 3]. Artificially mimicking the complex geometry of these networks comprising multiple branches and spanning several orders of magnitude in size represents a major hurdle in current biofabrication strategies. In particular, efforts have been made to reproduce the nature of capillary networks to improve nutrient supply in thick tissue substitutes.

In the past, artificial tissues have been restricted in size due to limited nutrient and oxygen supply in tissue depths over 200 µm [4, 5]. To overcome this size limitation, open channel structures that act as vascular networks can be incorporated, through which nutrient supply and removal of metabolic waste products can take place [6]. The inclusion of vascular, neuronal and lymphatic networks not only enables tissue constructs of clinically relevant sizes, but also adds the benefit of biomimetic substance transport through engineered tissue [7, 8]. In technical implementation, the production of such vascular, neuronal or lymphatic networks is a challenge, as the individual channel strands span several size scales from a few micrometers up to a few millimeters [9, 10]. Other challenges addressed in current research are the fine and ordered distribution of microvessels inside the engineered tissue [7], improving the resolution of biofabrication strategies [11], and fabrication speed [6]. However, cross-dimensional structuring at high-scale production speed has not been subject of previous research.

Nowadays, there are a variety of methods to include biological networks into engineered tissue [12]. The most common production techniques include the fabrication of molds using soft-lithography [13–15] and both direct [16–18] and indirect [19–21] 3D printing strategies. The aforementioned methods all require a form or template, such as a mask or pathway for the 3D printer, which defines the look or appearance of the networks in advance. Furthermore, the complexity and minimum size of these structures are limited by the resolution of the fabrication process. While soft lithography offers a tremendous resolution of down to a few micrometers in biofabricated vascular networks [22], it is a time-consuming process and requires expensive vacuum technology [23]. 3D bioprinting is particularly limited in terms of resolution; with most approaches limited to structures larger than 50 µm [24]. In both 3D printing and lithography, the area of network structures is usually limited to square centimeters or even square millimeters [12].

This leaves both research and industry in need of a rapid fabrication technology that can produce structured biological networks with sufficient resolution on the meter scale. Conventional, mass-production printing technologies like flexographic printing are well established technologies that combine fabrication speeds of meters per second with a resolution of e.g. down to sub-5 µm resolution in case of recently developed gravure printing cylinders [25]. Sterile or clean-room operating processes are also already established for electronic devices [26], point-of-care devices [27] and printed drug-delivery systems [28, 29]. However, they have not been in the focus of complex tissue engineering yet.

In our research, we could show that it is possible to use the conventional printing technology of flexographic printing to rapidly pattern substrates with several square centimeters of area with a sacrificial bioink with networks that mimic in shape and size the natural vessel network (figure 1). We achieved this by employing a well-known phenomenon in conventional printing technology called viscous fingering. Viscous fingering is a spontaneous pattern formation phenomenon which takes place during fluid transfer and appears on the printed product in form of finger-like structures [30] (figure 1). It is caused by the replacement of a higher viscous fluid by a lower viscous fluid and was first described by Saffman and Taylor in a generic Hele-Shaw-cell experiment [31]. Spontaneous pattern formation such as viscous fingering is usually considered a defect in graphical and functional printing, since it causes surface inhomogeneity [32]. However, we took advantage of its characteristics to print networks that mimic vascular networks in sacrificial gelatin. The process presented here requires no form or template and only a simple laboratory scale printing machine. The results are complex, connected networks with a tolerable size and distribution. The process is open to a variety of sacrificial printing materials and can, combined with casting of arbitrary hydrogel cells suspensions, produce versatile, vascularized engineered tissues at fabrication speed of meters per second.

2. Results and discussion

2.1. General concept of the bioprinting process

Viscous fingering can be observed in conventional printing technologies and leads to finger-like structures on the printed product, which are predominantly considered as a printing defect in graphical and functional printing. Its origin lies in the hydrodynamic fingering instability at the air-ink interface in the diverging printing nip during fluid transfer (figure 1(a)). When taking a closer look, viscous fingering in printing technologies forms intricate structures that greatly resemble vascular and lymphatic networks. This resemblance led us to the
Figure 1. Viscous fingering in flexographic printing (a) leads to finger-like structures on the printed product (b) which are connected, branched, stochastic and biomimetic. The printed finger-like structures are casted with a cell-laden hydrogel of choice (c), to fabricate perfusable tissue models. The finger-like structures resemble very much vascular and lymphatic networks as seen under the light microscope (d) and in profilometric images (e). This research shows that the printed structures are suitable for the fabrication of biological networks which will revolutionize future fabrication of tissue models towards large-scale mass production. Perfused casts of finger-like structures fabricated via plate-separation show the general concept (f).

The printed structures created in this research show a broad range of finger widths and distances, which are in the range of biological vascular structures spanning tens of micrometers up to a few millimeters (figure 1(b)). Additionally, the finger height is very suitable for vascular networks (figure 1(e)). In a next step, the printed, finger-like structures can be casted in a hydrogel of choice, laden with tissue-specific cells, to obtain perfusable tissue models (figure 1(f)).

2.2. Influence of printing parameters on the resulting network structures

The finger-like structures resulting from viscous fingering in flexographic printing span an order of magnitude. Adjusting the printing parameters, printing fluid rheology and the surface properties of the substrate, branching structures ranging from a few micrometers to a few millimeters can be produced. Although the printed finger-like structures...
look slightly different in every printing trial due to their stochastic nature, the average finger width, distance and height (figure 2) can reliably be reproduced for a certain set of printing parameters.

The printing machine comprises several components and different printing parameters, which all have an influence on the resulting fingering (figure 2, supporting information S2 available online at stacks.iop.org/BMM/17/045012/mmedia). However, our studies revealed that only three of them strongly influence the resulting finger width, height and distance of printed gelatin fingers. Thus, only those were systematically investigated in greater detail subsequently:

(a) Printing velocity \( v \)
(b) Anilox roller dip volume
(c) Shear viscosity \( \eta \) of printing fluid.

2.2.1. Influence of printing velocity

One of the three parameters, which strongly impacts the appearance of the printed finger-like structures (figure 3), is the printing velocity \( v \). This observation is in accordance with previous research on the impact of printing velocity on viscous fingering in flexographic printing [30, 34]. The capillary number \( Ca \), which is a dimensionless quantity representing the relation of viscous and surface tension forces, plays a big role in the phenomenon of viscous fingering [30, 31] and is defined as a characteristic velocity, in our case the printing velocity \( v \), multiplied by the shear viscosity \( \eta \) and divided by the surface tension \( \sigma \) of the fluid (equation (3)). Depending on the elasticity of the printing form, the influence of the capillary number \( Ca \) on finger formation is stronger or weaker. Quantitatively, the dominant finger distance \( \lambda \) is proportional to the capillary number \( Ca \) to the power of \( m \). For rigid printing forms as used in gravure printing and \( m = -0.5 \) for rigid printing forms as used in flexographic printing, respectively [30, 34] (equations (1) and (2)). This means that the dominant finger distance \( \lambda_{\text{rigid}} \) is proportional to the printing velocity \( v \) to the power of \( -0.5 \) in flexographic printing (equation (4)). When structured printing forms and special printing form setups are used, the dependency on the printing velocity might be broken, as shown for gravure printing in previous work [35, 36].

The dominant finger distance using rigid printing forms \( \lambda_{\text{rigid}} \) and the dominant finger distance using elastic printing forms \( \lambda_{\text{elastic}} \) are defined as:

\[
\lambda_{\text{rigid}} \propto Ca^{-0.5} \tag{1}
\]

\[
\lambda_{\text{elastic}} \propto Ca^{-0.1} \tag{2}
\]

The capillary number \( Ca \) is known as:

\[
Ca = \frac{v\eta}{\sigma} \tag{3}
\]

where \( v \) is the printing velocity, \( \eta \) is the shear viscosity and \( \sigma \) is the surface tension of the printing fluid. We thus obtain:

\[
\lambda_{\text{elastic}} \propto v^{-0.1} \tag{4}
\]

Figures 3(a) and (b) shows exemplary light microscope and profilometric images of finger-like structures on foil obtained at 0.2 m s\(^{-1}\) and 1.5 m s\(^{-1}\) which show clear differences in appearance, illustrating the influence of printing velocity. At 0.2 m s\(^{-1}\), the finger-like structures are more homogeneous than at 1.5 m s\(^{-1}\), where they are less connected and show more agglomerations of hydrogel. Inhomogeneities and reduced connectivity are both disadvantageous for the fabrication of vascular structures. Nevertheless, reduced printing velocities of 0.2–0.4 m s\(^{-1}\) in combination with the gelatin concentrations used in this work showed very good results for vascular network fabrication. Profilometric images confirm that the height of the fingers is in the desired range of two micrometers (figure 3(b)).

Interestingly, mean finger width does not exhibit a clear trend for a series of printed finger-like structures at different velocities from 0.2 to 1.5 m s\(^{-1}\) but fluctuates between 175 and 225 µm (figure 3(c)). In contrast, a slight decreasing trend of finger distance with increasing printing velocity is observable.
Figure 3. Influence of printing velocity on the printed finger-like structures as seen on light microscopic images (a) and profilometric images (b). At lower printing velocities, the finger-like structures appear more homogeneous whereas at high printing velocities, the structures are less connected and agglomerations of gelatin can be found. Finger width plotted over printing velocity (c) shows no clear trend whereas finger distance shows a slight downward trend with increasing printing velocity (d), as confirmed in a double logarithmic plot of finger distance over printing velocity (e). The linear fit with a coefficient of determination of $R^2 = 0.27$ obtains a slope of $m = −0.03$ which is a flatter slope than the theoretical prediction of $m = −0.1$.

(figure 3(d)) as predicted by theory. Mean finger distance ranges between 310 and 370 μm. However, the observed linear trend in the double logarithmic plot with a slope of $m = −0.03$ (coefficient of determination $R^2 = 0.27$) is a flatter slope than the theoretical prediction of $m = −0.1$ (equation (4)).

2.2.2. Influence of anilox roller dip volume
The second parameter that strongly affects the appearance of the printed finger-like structures is the anilox roller dip volume (figure 4). The anilox roller is covered with engraved microscopic cells, which are filled with printing fluid by dipping them into a fluid reservoir and are then pressed against the printing form. In this way, the anilox roller transfers the printing fluid onto the printing form. The shape and size of the cells defines the so-called dip volume of the anilox roller, which stands for the maximum amount of ink transferred to the printing form. For the printed finger-like structures, an increased dip volume results in increased average finger width, height and distance (figures 4(c) and (d)). For foil, mean finger widths between 50 and 80 μm, mean finger heights between 0.75 and 1.75 μm and mean finger distances between 200 and 300 μm can be reached just by variation of the dip volume. For glass, mean finger widths between 40 and 70 μm, mean finger heights between 0.5 and 1.25 μm and mean finger distances between 250 and 300 μm can be achieved in the same way. The highest values for finger width, height and distance apply for the highest dip volume of 16 ml m$^{-2}$.

The influence of the dip volume has already been shown for viscous fingering in gravure printing [36, 37]. In flexographic printing, a higher dip volume leads to a higher transferred fluid volume to the printing cylinder and thus to a higher amount of ink transferred to the substrate. This results in finger-like structures with a higher volume to area ratio, resulting in larger-spaced, broader and higher finger-like structures in case of our research (supporting information S3). Since vascular networks require homogeneous, fine finger-like structures with
Figure 4. The anilox roller dip volume also has an influence on the appearance of the finger-like structures. This is demonstrated exemplarily with profilometric images of finger-like structures on glass (a), caused by shape and size of the cells (b), which cover the anilox roller. In case of lower dip volumes, the cells are smaller and shallower whereas for higher dip volumes, the cells are larger and deeper. Finger width, height and distance over anilox roller dip volume for foil (c) and glass (d) all show an increasing trend with increasing dip volume. Also see supporting information S3 for images that illustrate the influence of dip volume on finger distribution.

a stable distribution and certain minimum height, the dip volume should not be too small but also not too large when gelatin is used as printing fluid. The optimum dip volume for the laboratory printing machine employed in this work was 8 ml m\(^{-2}\).

2.2.3. Influence of shear viscosity

The shear viscosity of the printing fluid had the greatest influence on the resulting structures of all studied parameters (figure 5). In our case, shear viscosity was directly governed by the gelatin concentration. For example, a gelatin gel with a 10% concentration had a shear viscosity of about 23 mPas and a 30% gelatin gel of about 700 mPas, exhibiting a slight shear thinning behavior (figure 5(b) and see supporting information S4 for all viscosity curves). The shear viscosity in mPas over gelatin concentration in % could be fitted with an exponential fit curve \(y = -3.74 + 5.85e^{0.15x}\) (figure 5(c)). High gelatin concentrations of about 25%–30% resulted in the formation of distinct, closely spaced, biomimetic networks, while with low concentrations of approx. 5%–10% only broad, randomly connected regions could be achieved ((figure 5(a) and supporting information S4)). For foil, mean finger widths range from 50 to 350 \(\mu\)m, mean finger heights from 0.15 to 1.25 \(\mu\)m, and mean finger distances from 200 to 500 \(\mu\)m (figure 5(d)). For glass, mean finger width lie between 50 and 350 \(\mu\)m, mean finger heights between 0.1 and 1.0 \(\mu\)m and mean finger distances between 250 and 500 \(\mu\)m (figure 5(e)). The smallest finger widths and distances and the greatest finger heights could be achieved with highest gelatin concentrations.

In a previous work, a mathematical model for fluid transfer in flexographic printing based on the hydrodynamic lubrication theory \cite{34} was presented. Interestingly, this work demonstrated that the results obtained for the printing of gelatin hydrogels are in line with the theory \cite{30}, which states that the dominant finger distance for elastic printing forms \(\lambda_{\text{elastic}}\) is proportional to the fluid shear viscosity \(\eta\) to the power of \(m = -0.1\) \cite{34} (equation (5)), which can be derived from equation (2).

\[
\lambda_{\text{elastic}} \propto \eta^{-0.1}.
\] (5)

This means that with higher printing fluid shear viscosity, the finger distance decreases, resulting in
Figure 5. Influence of gelatin concentration. Microscopic and profilometric images show great difference in appearance of the finger-like structures when using different gelatin concentrations for printing (a). Exemplarily, shear viscosities of 10% and 30% gelatin gel are plotted over shear rate (b). Also see supporting information S4. The averaged shear viscosities of all gelatin gels used for printing are given over gelatin concentration (c). An exponential fit curve is presented in the plot. Measurements of finger width, finger height and finger distance for foil (d) and glass (e) over gelatin concentration confirm the influence of viscosity on the printed finger-like structures. When plotting average finger distance for different shear viscosities in double logarithmic scale for foil (f) and glass (g), the linear fit for both substrates obtains a slope of \( m = -0.13 \) which suits very well to the theoretical prediction of \( m = -0.1 \). The coefficients of determination are \( R^2 = 0.63 \) for foil and \( R^2 = 0.86 \) for glass, thus the quality of the linear fit is relatively good for both substrates.

more closely spaced networks. This is in accordance with the findings of this research. When plotting average finger distance for different shear viscosities in double logarithmic scale for foil (figure 5(f)) and glass (figure 5(g)), the linear fit for both substrates obtains a slope of \( m = -0.13 \) which suits very well to the theoretical prediction of \( m = -0.1 \). The coefficients of determination are \( R^2 = 0.63 \) for foil and \( R^2 = 0.86 \) for glass, thus the quality of the linear fit is relatively good for both substrates.
In addition to the aforementioned lubrication theory, the surface levelling behavior of the finger-structures after printing must also be taken into account here [38]. In the case of low viscous gelatin, the printed, largely spaced network structures end up in broad, ink-covered regions due to fast spreading of the gelatin over large areas of the substrate before gel-ling. In case of the highly viscous gelatin, the spreading is very slow due to inner friction forces and does not lead to a significant broadening of the finger-like structures before gelling. Therefore, highly viscous gelatins with either a high Bloom number or at high concentrations (25%–30%) are most suitable for the fabrication of vascular networks.

2.2.4. Influence of the printing substrate

Various substrates can be considered in flexographic printing, as there are no limits to the material with this technology. In this work, the influence on finger formation of two transparent materials commonly used in biofabrication, glass and PET foil, were studied. Glass is the gold-standard in cell culture, microbiology and microscopy, as it is biologically compatible, can easily be sterilized and offers high transparency. PET foil is a much more flexible material with different wetting properties, which offers enormous scalability as it can be handled in a roll-to-sheet process. The choice of substrate influenced the shape of fingers in different ways. While networks printed on foil appear more branched (figures 6(a) and (b)), the structures become more aligned when printed on glass (figures 6(c) and (d)). This is probably caused by the different elasticity of the substrates, which influences viscous finger formation [34]. While the foil used in this research is deformable and might be slightly lifted from the substrate carrier during fluid transfer, the glass substrate is rigid and does not deform during printing. With 13 mN, the bending resistance for the foil substrate was determined as at least three orders of magnitude lower than the bending resistance of the glass substrate. Another reason for the different appearance of the finger-like structures on foil and glass probably is the wetting behavior of the gelatin on the substrates. For gelatin concentrations between 0% and 25%, gelatin showed a much higher contact angle on foil than on glass (figures 6(e) and (f)). Only for a 30% concentration, contact angles were similar for glass and foil, around 60°.

2.2.5. Additional printer components and parameters

As indicated in the first part of this chapter, additional components and parameters of the printing machine, such as printing force, anilox force, anilox roller temperature and influence of cleaning procedure, were studied. However, those were shown to have only little impact on finger shape and size (supporting inform-ation S2, S5–S7). For optimized printing results, we therefore adjusted these parameters towards enabling most simple handling of the device and achieving a reliable printing process.

2.2.6. Process optimization towards the biofabrication of vascular networks

Following the systematic investigation of the previously described printing parameters the process was finally adjusted towards biofabrication of vascular networks. For fabricating vascular networks, we assumed that finger widths should not be larger than 50 µm and finger height should be larger than 1 µm to enable the insertion of endothelial cells. Besides, fin-ger distance should be smaller than 400 µm to provide a good supply of nutrients and avoid necrosis. The printing parameters that led to results most suitable for vascular networks regarding finger width, height and distance are a gelatin concentration of 30%, an anilox roller dip volume of 8 ml m⁻², an anilox force of 50 N, a printing force of 10 N and a printing velocity of 0.2 m s⁻¹ for foil and 0.4 m s⁻¹ for glass (table 1). Exemplary printing results with optimized parameters are shown in figure 7. Although printed finger-like structures look different on foil and glass (figures 7(a) and (b)), resulting finger width, height and distance are quite similar. When utilizing optimized printing parameters, mean finger width lies around 40–50 µm, mean finger height around 0.7–1.25 µm and mean finger distance around 150–200 µm (figure 7(c)). This fulfills the requirements for biofabrication of vascular networks.

In comparison to so far investigated 3D bioprinting approaches, such as drop-on-demand bioprinting or microextrusion, that yield channel structures of about 100 µm in diameter [12], the finger-like hydrogel structures created with flexographic printing enable a much higher lateral and vertical (approx. 1 µm) resolution. Thus, they enable the production of capillary networks with a width of 40–50 µm. In addition, the optimized printed network structures in this work are finely distributed with finger dis-tances around 200 µm on average, therefore ensur-ing a sufficient nutrient and oxygen support of tissue between the capillary network. Of course, with soft-lithography [39], stereolithography and DLP [40, 41], two-photon-polymerization [42] or recently reported photo-ablation procedures [43], comparably small dimensions can be achieved, however, so far this is only possible at much higher fabrication times ranging from several minutes to hours. Using the laboratory-scale printing process as utilized in this research, the fabrication of networks structures as shown in figures 7(a) and (b) of size 25 000 mm² takes place within about 2.5 s. Even more, the net-works structures can be reproducibly printed and flexibly adjusted to the shape and size desired for a specific application. Other porogenic materials such as poloxamers, and even other thermally or long-time gelling materials, can be structured using this approach. Viscous fingering in conventional printing
Figure 6. Influence of substrate on finger shape. Finger networks printed on foil are more branched (a) and (b) whereas they appear more aligned on glass (c) and (d). This might be caused by the distinct wetting behavior of gelatin on foil (e) and on glass substrates (f). We plotted contact angle over gelatin concentration, with 0% concentration meaning the wetting of pure distilled water for comparison. Another reason for the different finger shape on glass and foil might be the different elasticity of the substrates.

Table 1. Optimized printing parameters for foil and glass.

| Substrate | Gelatin concentration in % | Anilox roller dip volume in ml m$^{-2}$ | Anilox force in N | Printing force in N | Printing velocity in m s$^{-1}$ |
|-----------|-----------------------------|----------------------------------------|-------------------|---------------------|------------------------------|
| Foil      | 30                          | 8                                      | 50                | 10                  | 0.2                          |
| Glass     | 30                          | 8                                      | 50                | 10                  | 0.4                          |
technologies is therefore a great platform for structuring biomaterials over a broad order of magnitude and for diverse applications in biofabrication.

2.3. Embedding the printed vascular networks in hydrogel bulk matrices
To demonstrate that the printed finger-like structures are suitable master molds for vascular networks, printed structures were casted in agarose as a model material for future perfusable tissue models. By casting, a complete and exact replica mold of the finger-like structures was obtained as shown in figure 8. The casts showed finger widths of about 50 µm and finger heights of 3–5 µm, which were ideally suited for the fabrication of vascular networks. Interestingly, the size of the replicas were larger than in the printed finger-like structures (figure 8). This could be attributed to the repulsive surface charge of gelled gelatin structures and the formation of a thin aqueous layer at the gelatin-agarose interface, which we observed. The detachment of the casted hydrogel from the printed mold was easily achieved and no thermal melting of gelatin was required. This process is applicable to hydrogels, of both natural and synthetic origin, and with concentrations tailored to the tissue of choice, making the printed finger-like structures a versatile, platform technology for the biofabrication of arbitrary, vascularized tissue.

Figure 7. Exemplary samples printed with optimized printing parameters on foil (a) and glass (b). This leads to optimized finger width, height and distance as given in (c).

Figure 8. Cast of finger-like structures in agarose showing an exact replication of the network structure (a) with increased finger dimensions (b) compared to the printed finger masters.
In sum, the fabricated finger-like structures were highly branched, inter-connected, and showed a stochastically distributed, biomimetic morphology. In particular, their high-level of interconnectivity renders them suitable for perfusion of the casted hydrogel replicas. As a pre-test for future perfusion, a droplet of dyed water was infiltrated into a similarly produced network created in a plate-separation test (figure 1(f)). In conclusion, the presented method enables large-scale, fast, and cost-efficient fabrication of perfusable tissue constructs at industrial scale.

3. Summary and outlook

In this paper, the fabrication of highly branched, dendritic conduit networks using flexographic printing and the phenomenon of viscous fingering was shown for the first time. The presented approach holds great promise for the large-scale and time- as well as cost-efficient biofabrication of hierarchically organized channel networks. In the form of blood vessels as well as lymphatic or neural networks, these highly organized structures make a decisive contribution to the exchange of substances and information between tissues and organs and thus to the functionality of complex organisms. In an initial feasibility study, the application of the described printing process was demonstrated on the example of channel structures that mimic the morphology of capillary vessels. The dimensions of the produced finger-like structures could precisely be adjusted through systematic variation of the printing velocity, the anilox roller dip volume, and the shear viscosity of the applied printing fluid. Best results of the finger-like structures were achieved at a gelatin concentration of 30% with a viscosity of 700 mPas, an anilox roller dip volume of 8 ml m⁻² and at comparably low printing velocities (0.2–0.4 m s⁻¹). In the optimized printing experiments, finger widths ranging from 35 to 50 µm could be achieved with a vertical resolution of 0.5–1.3 µm. These highly branched networks could be biofabricated with a laboratory scale printing machine on an area of about 50 × 500 mm² within just 2.5 s, resulting in yet unprecedented printing velocities (10,000 mm² s⁻¹) and print volume flows (10 mm³ s⁻¹). The high printing output renders the presented method useful for high-scale and ultra-high-speed fabrication of hierarchically organized tissues. The subsequent casting of the samples with agarose lead to finger widths of about 50 µm and slightly larger finger heights of 3–5 µm, both beneficial for the recreation of e.g. vascular networks.

While other biofabrication methods, such as soft-lithography [39], stereolithography and DLP [40, 41], two-photon-polymerization or recently reported photo-ablation procedures [43] enable similar resolutions, flexographic printing outperforms in terms of its unprecedented bioprinting efficiency, which is achieved in two ways:

(a) First of all, its innate nature to create highly branched, dendritic networks with controllable size and distance, but stochastically distribution, renders the need for time consuming printing preparation procedures, including algorithms to calculate ideal capillary network distribution, 3D modelling and slicing, unnecessary.

(b) Second, the printing procedure itself enables high scale and ultra-high-speed fabrication of the before mentioned networks reaching printing velocities of several 10,000 mm² s⁻¹ and print volume flows of several 10 mm³ s⁻¹.

Another advantage of the developed method is its broad applicability, which is not limited to the fabrication of vascular networks only. Due to the adjustable dimensions, distances and branching factors that could be achieved as well as the broad range of sacrificial hydrogels, the method holds great promise for the rapid fabrication of networks with patterns related to e.g. neural or lymphatic morphology, too. Furthermore, casting and subsequent detachment of hydrogel matrices onto the printed network were shown to be simple and reliable and could be demonstrated without damaging the finger geometries. Analog to already established casting methods, such as soft-lithography, the developed method enables the reproduction of finger-like structures applying versatile matrix materials, such as agarose, collagen, GelMa or Matrigel.

In summary, the process described is characterized by its high efficiency, versatility and wide range of applications. Not only can industrial printing machines be used for fabrication of biological networks, but there are also affordable and easy-to-use laboratory scale printing machines on the market that make the revolutionary technology of printing biological networks for tissue engineering, as presented in this research, available for small laboratories, research institutions or hospitals. The choice of substrate, hydrogel and type of cells is arbitrary and thus can be tailored according to the specific needs of the user or the chosen tissue model (liver model, heart model, etc.). In subsequent work, the biologization of the generated structures as well as the transferability to other biological channel structures and conductive pathways, such as those found in lymphatic and neuronal networks should be examined. A comparison using fractal dimensions of the printed networks to these biological networks would also offer a further criterion for the selection of optimum parameters. Furthermore, the applicability of the method to the fabrication of multifunctional tissues for regenerative medicine and organ-on-a-chip.
systems will be investigated. The first results on the wettability and perfusion of the channels (see figure 1(f)) offer a foretaste of promising research and development opportunities.

4. Experimental

4.1. Printing process

The printing experiments were conducted with an IGT F1 flexographic printing machine (IGT Testing Systems, Almere, the Netherlands), see figure 9. The machine is a small (300 × 650 × 400 mm$^3$), easy-to-use, affordable printing machine normally used as a printability tester in the laboratory. Differences to conventional flexographic machines are the force-controlled pressure and the manual application of fluid to the anilox roller.

The basic process of flexographic printing as well as the laboratory printing machine used in this research are shown in figure 9. The anilox roller, which is structured with very small micrometer-sized cells, dips into the ink reservoir and the cells fill with printing fluid. Excess fluid is wiped off by the doctor blade. The anilox roller completely wets the flexible relief-like printing form. The anilox roller’s cell volume per area, the dip volume, determines the fluid volume that is provided to the printing form. The printing form transfers the fluid to the substrate, which is pressed against the printing form by the impression cylinder. Industrial flexographic printing machines can operate at printing velocities up to about seven meters per second [33].

As printing form, we used an unstructured full-area, photo polymeric, flexographic printing form (50 × 500 × 1.14 mm$^3$, nyloflex FAH Digital, ordered via Repro-Form Druckvorlagen und Gravur GmbH, Dieburg, Germany). It was attached to the cylinder with a double-sided adhesive tape (DuploCOLL®, Lohmann, Neuwied, Germany). As substrates, we used a 125 μm thick transparent polyethylene terephthalate (PET) foil (Hostaphan® GN 4600/125 μm, Pütz Folie, Taunusstein, Germany) of size 60 × 750 mm$^2$ and soda-lime glass carriers of size 50 × 50 × 1 mm$^3$ (Glaswarenfabrik Karl Hecht, Sondheim/Rhoen, Germany).

Several test series on foil and glass were carried out, where one parameter was varied while the other parameters were kept constant. Gelatin concentration was varied from 5% to 35%, anilox roller dip volume from 4.5 to 24 ml m$^{-2}$, anilox force as well as printing force from 10 to 500 N and printing velocity from 0.2 to 1.5 m s$^{-1}$ (table 2).

4.2. Hydrogel preparation

Different hydrogels at several concentrations with and without dye were prepared. For the printing experiments, gelatin gels and agarose gels were prepared for the printing and casting experiments, respectively.

The gelatin gels that were applied during flexographic printing were prepared using type A gelatin powder (G1890, Sigma-Aldrich, St. Louis, USA), made from porcine skin with a Bloom value of 300 g. We prepared gels in distilled water with varying gelatin concentrations (5, 10, 15, 20, 25, 30, 35 wt/v%). The red food dye ‘New Coccine’ (199737, Sigma-Aldrich, St. Louis, USA) was added to the prepared gelatin gels to make the printed finger-like structures clearly visible to the observer and to enhance contrast during microscopic analysis. The dye concentration was kept constant for all gels at 2.5 wt/v%. The gelatin gels were heated in a water bath at 40 °C for 15 min until they formed a homogeneous fluid ready for printing.

The agarose gel used for casting experiments was prepared with agarose powder (A9414, Sigma-Aldrich, St. Louis, USA) at 2 wt/v% in distilled water without dye. The agarose gel was heated to 90 °C in a water bath until the agarose powder was completely

| Parameter                        | Value range |
|----------------------------------|-------------|
| Substrate                        | Foil, glass |
| Gelatin concentration in %       | 5–35        |
| Anilox roller dip volume in ml m$^{-2}$ | 4.5–24     |
| Anilox force in N                | 10–500      |
| Printing force in N              | 10–500      |
| Printing velocity in m s$^{-1}$  | 0.2–1.5     |

Figure 9. Process of flexographic printing: Schematic representation (a) and laboratory printing machine (b). See video of the printing process in supporting information S1.
solved, then the temperature of the water bath was decreased to 40 °C for 10 min until the agarose gel was ready to use for casting experiments.

In this paper, % is used as a synonym for wt/v%.

### 4.3. Hydrogel characterization

We measured the shear viscosity of the used dyed gelatin gels with a rotary oscillating rheometer (Kinexus lab+, NETZSCH-Gerätebau, Selb, Germany), using a 1° cone-plate geometry with diameter of 60 mm. The shear viscosity was measured for shear rates from $1 \text{s}^{-1}$ to $3000 \text{s}^{-1}$ at 40 °C, taking two measurement points per decade. For each gelatin gel formulation, three shear rate curves were taken. Mean value and standard deviation of shear viscosity were plotted for every measured shear rate.

Wetting behavior of all dyed gelatin gels was measured using a contact angle measuring device (DSA 100, Krüss, Hamburg, Germany) using the sessile drop method. For gelatin gels with 5%, 10% and 15% concentration, droplets with a volume of 1 µl were measured, for 20%, 25% and 30%, the droplet volume was increased to 10 µl to improve the handling of the more viscous material. Per gelatin gel formulation and per substrate, six sessile droplets were measured, taking ten measurements per droplet.

### 4.4. Characterization of network structures

The appearance of the printed finger-like structures was evaluated visually using an upright microscope in bright field mode (DM4000M, Leica, Wetzlar, Germany). At least two images were taken for each printed sample at locations that were as homogeneous as possible. At least five to ten finger widths were measured in each image, including thin as well as thick structures.

The finger distance was acquired in MATLAB using one dimensional fast Fourier transformation perpendicular to the finger direction as utilized by our group in a similar way before [30]. For each pixel row of a light microscope image, the finger frequency was determined by finding the amplitude peak of the frequency spectrum in a predefined frequency range, which was chosen according to the expected minimum and maximum finger frequencies. The finger frequency was converted into a finger distance according to given pixel size and absolute resolution of the input image. This led to 1960 measuring points of finger distance per image.

The finger height was analyzed with a 3D optical profilometer (Sensofar S NEOX, Sensofar, Barcelona, Spain) in confocal mode with a $20 \times$ EPI objective (Nikon, Minato, Japan). Single images as well as

---

### Table 3. Printing parameters used to obtain images for this research.

| Figure | Substrate | Gelatin concentration in % | Anilox roller dip volume in ml m$^{-2}$ | Anilox force in N | Printing force in N | Printing velocity in m s$^{-1}$ |
|--------|-----------|---------------------------|-----------------------------------------|------------------|-------------------|-----------------------------|
| Figure 1(d) | Glass | 30 | 8 | 50 | 300 | 0.2 |
| Figure 1(c) | Glass | 30 | 8 | 50 | 10 | 0.4 |
| Figure 3(a) | Foil | 30 | 8 | 50 | 20 | 0.2, 1.5 |
| Figure 3(b) | Foil | 30 | 8 | 50 | 20 | 0.2, 1.5 |
| Figure 3(c) | Foil | 15 | 16 | 50 | 20 | 0.2, 0.3, 0.4 ... 1.5 |
| Figure 3(d) | Foil | 15 | 16 | 50 | 20 | 0.2, 0.3, 0.4 ... 1.5 |
| Figure 3(e) | Foil | 15 | 16 | 50 | 20 | 0.2, 0.3, 0.4 ... 1.5 |
| Figure 4(a) Left | Glass | 30 | 4.5 | 50 | 20 | 0.4 |
| Figure 4(a) Right | Glass | 35 | 16 | 50 | 20 | 0.4 |
| Figure 4(c) | Foil | 30 | 7, 9, 11, 16 | 50 | 20 | 0.4 |
| Figure 4(d) | Glass | 30 | 7, 9, 11, 16 | 50 | 20 | 0.4 |
| Figure 5(a) Left top | Foil | 10 | 16 | 50 | 20 | 0.5 |
| Figure 5(a) Left bottom | Foil | 10 | 8 | 50 | 20 | 0.4 |
| Figure 5(a) Right top | Foil | 30 | 16 | 50 | 20 | 0.5 |
| Figure 5(a) Right bottom | Foil | 30 | 8 | 50 | 20 | 0.4 |
| Figure 5(d) | Foil | 5, 10, 15, ... 30 | 16 | 50 | 20 | 0.5 |
| Figure 5(e) | Glass | 5, 10, 15, ... 30 | 16 | 50 | 20 | 0.5 |
| Figure 5(f) | Foil | 5, 10, 15, ... 30 | 16 | 50 | 20 | 0.5 |
| Figure 5(g) | Glass | 5, 10, 15, ... 30 | 16 | 50 | 20 | 0.5 |
| Figure 6(a) | Foil | 30 | 8 | 50 | 10 | 0.2 |
| Figure 6(b) | Foil | 25 | 16 | 50 | 20 | 0.5 |
| Figure 6(c) | Glass | 30 | 8 | 50 | 10 | 0.2 |
| Figure 6(d) | Glass | 25 | 16 | 50 | 20 | 0.5 |
| Figure 7(a) | Foil | 30 | 8 | 50 | 10 | 0.2 |
| Figure 7(b) | Glass | 30 | 8 | 50 | 10 | 0.4 |
| Figure 7(c) Foil | Foil | 30 | 8 | 50 | 10 | 0.2 |
| Figure 7(c) Glass | Glass | 30 | 8 | 50 | 10 | 0.4 |
2 × 2 stitched images with a size of approximately 1500 × 1250 μm² were acquired. At least one image was acquired for each printed sample. At least five to ten finger heights were measured per image.

4.5. Casting in hydrogel
Printed samples on glass were prepared with 30% gelatin, anilox roller dip volume 8 ml m⁻², anilox force 50 N, printing force 10 N, printing velocity 0.4 m s⁻¹ and then immediately cooled down in a refrigerator at 4.8 °C or a freezer at −20 °C. The prepared agarose gel for casting was poured onto the cold printed samples. The printed samples were manually removed after sol-gel transition of the applied agarose gel.

4.6. Measurement of substrate bending force
The bending resistance of the foil and the glass substrate was determined using a bending resistance tester (K416, Messmer Buchel, Veenendaal, the Netherlands) at a bending angle of 5° and a measuring distance of 25 mm. Ten measurements were taken for the foil substrate, using a new sample for each measurement. For the glass substrate, only three measurements were performed, since the bending resistance exceeded the limits of the load cell, which was 10 N.

4.7. Printing parameters used in figures
To be able to reproduce the results from this research, printing parameters used to create images for all figures in this paper are depicted in table 3.

Data availability statement
All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments
We kindly acknowledge the financial support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Project-ID 265191195—SFB 1194 ‘Interaction between Transport and Wetting Processes’, Project C01.

Conflict of interest
There are no conflicts to declare.

ORCID iDs
Pauline Brumm https://orcid.org/0000-0002-8220-0676
Anna Fritschen https://orcid.org/0000-0001-9114-8630
Lara Doß https://orcid.org/0000-0002-6796-7385
Edgar Dörsam https://orcid.org/0000-0002-4338-1777

References
[1] Carmeliet P and Tessier-Lavigne M 2005 Common mechanisms of nerve and blood vessel wiring Nature 436 193–200
[2] Abbott R D and Kaplan D L 2015 Strategies for improving the physiological relevance of human engineered tissues Trends Biotechnol. 33 401–7
[3] Mafai I, Kaur G, Seyedsalehi A, McClinton A and Laurencin C T 2020 Progress in 3D bioprinting technology for tissue/organ regenerative engineering Biomaterials 226 119536
[4] Jain R K, Au P, Tan J, Duda G D and Fukumura D 2005 Engineering vascularized tissue Nat. Biotechnol. 23 821–3
[5] Rouwkema J, Rivron C N and van Blitterswijk C A 2008 Vascularization in tissue engineering Trends Biotechnol. 26 434–41
[6] Paulsen S J and Miller J S 2015 Tissue vascularization through 3D printing: will technology bring us flow? Dev. Dyn. 244 629–40
[7] Rouwkema J and Khademhosseini A 2016 Vascularization and angiogenesis in tissue engineering: beyond creating static networks Trends Biotechnol. 34 733–45
[8] Bae H, Puranik A S, Gauvin R, Edalat F, Carrillo-Conde B, Peppas N A and Khademhosseini A 2012 Building vascular networks Sci. Transl. Med. 4 160ps23
[9] Schöneberg J, De Lorenzi F, Theek B, Blaeser A, Rommel D, Kuehne A J G, Kießling F and Fischer H 2018 Engineering biofunctional in vitro vessel models using a multilayer bioprinting technique Sci. Rep. 8 1–13
[10] Breslin J et al 2019 Vascular networks for tissue engineering and cardiovascular disease Phys. Med. Biol. 64 207–29
[11] Dutta P, Ayan B and Ozbolat I T 2017 Bioprinting for vascular and vascularized tissue biofabrication Acta Biomater. 51 1–20
[12] Fritschen A and Blaeser A 2020 Biosynthetic, biomimetic, and self-assembled vascularized organ-on-a-chip systems Biomaterials 268 120556
[13] Mao M et al 2020 Human-on-leaf-chip: a biomimetic vascular system integrated with chamber-specific organs Small 16 2000546
[14] Fenech M, Girod V, Claveria V, Meance S, Abkarian M and Charlot B 2019 Microfluidic blood vasculature replicas using backside lithography Lab Chip 19 1096–106
[15] Zheng Y et al 2012 In vitro microvessels for the study of angiogenesis and thrombosis Proc. Natl Acad. Sci. USA 109 9342–7
[16] Grigoryan B et al 2019 Multivascular networks and functional intravascular topologies within bio compatible hydrogels Science 364 458–64
[17] Jia W et al 2016 Direct 3D bioprinting of perfusable vascular constructs using a blend bioink Biomaterials 106 58–68
[18] Zhu W et al 2018 Direct 3D bioprinting of prevascularized tissue constructs with complex microarchitecture Biomaterials 124 106–15
[19] Noor N et al 2019 3D printing of personalized thick and perfusable cardiac patches and valves Adv. Sci. 6 1900344
[20] Lin N Y C, Homan K A, Robinson S S, Kolesky D B, Duarte N, Moisan A and Lewis J A 2019 Renal reabsorption in 3D vascularized proximal tubule models Proc. Natl Acad. Sci. USA 116 5399–404
[21] Kolesky D B, Homan K A, Skylar-Scott M A and Lewis J A 2016 Three-dimensional bioprinting of thick vascularized tissues Proc. Natl Acad. Sci. USA 113 3179–84
[22] Golden A P and Tien J 2007 Fabrication of microfluidic hydrogels using molded gelatin as a sacrificial material Lab Chip 7 720–9
[23] Xia Y and Whitesides G M 1998 Soft lithography Angew. Chem., Int. Ed. 37 550–75
[24] Blaeser A, Duarte Campos D F and Fischer H 2017 3D bioprinting of cell-laden hydrogels for advanced tissue engineering Curr. Opin. Biomed. Eng. 2 58–66
[25] Grau G et al 2016 Gravure-printed electronics: recent progress in tooling development, understanding of printing physics, and realization of printed devices Flex. Print. Electron. 1 023002
[26] Raupp S, Daume D, Tekoglu S, Merklein L, Lemmer U, Hernandez-Sosa G, Sauer H M, Dörsam E, Scharfer P and Schabel W 2017 Slot die coated and flexo printed highly efficient SMOLEDs Adv. Mater. Technol. 2 1600230
[27] Assaifan A K, Al Habis N, Ahmad I, Alshehri N A and Alharbi H F 2020 Scaling-up medical technologies using flexographic printing Talanta 219 121236
[28] Sandler N and Preis M 2016 Printed drug-delivery systems for improved patient treatment Trends Pharmacol. Sci. 37 1070–80
[29] Janßen E M, Schliephacke R, Breitenbach A and Breitkreutz J 2013 Drug-printing by flexographic printing technology—a new manufacturing process for orodispersible films Int. J. Pharm. 441 818–25
[30] Brumm P, Sauer H and Dörsam E 2019 Scaling behavior of pattern formation in the flexographic ink splitting process Colloids Interfaces 3 37
[31] Saffman P G and Taylor G I 1958 The penetration of a fluid into a porous medium or Hele-Shaw cell containing a more viscous liquid Proc. R. Soc. A 245 312–29
[32] Hernandez-Sosa G et al 2013 Rheological and drying considerations for uniformly gravure-printed layers: towards large-area flexible organic light-emitting diodes Adv. Funct. Mater. 23 3164–71
[33] Kipphan H 2001 Printing technologies with permanent printing master Handbook of Print Media ed H Kipphan (Berlin: Springer) [https://doi.org/10.1007/978-3-540-29900-4_2]
[34] Sauer H M, Daume D and Dörsam E 2015 Lubrication theory of ink hydrodynamics in the flexographic printing nip J. Print Med. Technol. Res. 4 163–72
[35] Schäfer J, Roisman I V, Sauer H M and Dörsam E 2019 Millisecond fluid pattern formation in the nip of a gravure printing machine Colloids Surf. A 575 222–9
[36] Schäfer J K 2020 In situ Untersuchung der hochdynamischen Grenzflächeninstabilität im Zylinderspalt Dissertation Technical University of Darmstadt [https://doi.org/10.25534/tuprints-00014204]
[37] Brumm P, Weber T E, Sauer H M and Dörsam E 2021 Ink splitting in gravure printing: localization of the transition from dots to fingers J. Print Med. Technol. Res. 10 81–93
[38] Sauer H M, Braig F and Dörsam E 2021 Leveling and drying dynamics of printed liquid films of organic semiconductor solutions in OLED/OPV applications Adv. Mater. Technol. 6 2000160
[39] Zhang B et al 2016 Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis Nat. Mater. 15 669–78
[40] Zhu W et al 2017 Direct 3D bioprinting of prevascularized tissue constructs with complex microarchitecture Biomaterials 124 106–15
[41] Huang T Q, Qu X, Liu J and Chen S 2014 3D printing of biomimetic microstructures for cancer cell migration Biomol. Microdev. 16 127–32
[42] Marino A et al 2018 A 3D real-scale, biomimetic, and biohybrid model of the blood-brain barrier fabricated through two-photon lithography Small 14 1702959
[43] Brandenberg N and Lutolf M P 2016 In situ patterning of microfluidic networks in 3D cell-laden hydrogels Adv. Mater. 28 7450–6