

Natural polymer hydrogel based 3D printed bioreactor testing platform for cancer cell culture

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N ur l polym r hydrog l b s d 3D prin d bior c or s ing pl form for c nc r c ll cul ur

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ABSTRACT

Al hough wo-dimension l c ll cul ur s provid ime nd cos - ff c iv m e h o d f o r s i n g d r u g s h p r c l i n i c i l l v l , h y d o n o c p u r h h r - d i m e n s i o n l c l l u l r i n r c i o n s o r u m o r p n r i o n h m u s o c c u r i n v i v o . T h r f o r , h s d r u g s o f n f i l s h y r n s i o n f r o m w o - d i m e n s i o n l c u l u r m o d l s o m o r c o m p l x o n s , i n c l u d i n g n i m a l m o d l s o r v n h u m a n c l i n i c l r i l s . T h g o l o f h i s r s r c h w a s o d v l o p h r - d i m e n s i o n l b i o p r i n d p n e r i c c n e r d r u g s i n g p l f o r m h c o u l d i n c r s h f f c i v n s s o f d r u g s i n g d u r i n g h r l y p r c l i n i c l s g s . S p c i f i c l l y , d i f f r n n u r l p o l y m r h y d r o g l f o r m u l i o n s o f c l l u l o s , l g i n , n d g l i n w e r s d o f i n d h o p i m a l p r i n b i l i y n d c l l v i b i l i y . I w a s d r m i n d h c l l u l o s n n o c r y s l n d l g i n h y d r o g l p r o v i d d h b s p r i n b i l i y b c u s o f i s s u p r i o r s h r h i n n i n g p r o p r i s . I n d d i o n , B x P C - 3 c l l s , h w e r p r i n d n d h n c u l u r d w i h i n h i s h y d r o g l f o r f o u r d y s , x h i b i d r n g o f c l l v i b i l i y b w e n 8 % n d 6 % . T o s i m u l v s c u l u r r o u n d h b i o p r i n d c u l u r s , s p i n n i n g b i o r c o r w a s m a n u f c u r d ; h o w e r , i d i s u r b d h c l l s , l d i n g o d c r s i n c l l v i b i l i y c o m p r d o s g n n c u l u r s . O v r l l , h p l f o r m d m o n s r d g o o d p r i n b i l i y n d c l l v i b i l i y f o r f u u r u s i n p n e r i c c n e r d r u g s i n g .

1. Introduction

C nc r c c o u n d f o r n s i m a d 9.6 m i l l i o n d h s w o r l d w i d i n 2 18 [1]. W i h n o h r 18.1 m i l l i o n n w d i g n o s s i n h s m e y r , h n d f o r n w r m e n s n d h r p i s i s s g r s v r [1]. I n U n i d S l o n , b o u 6 9,82 c n c r d h s w a s p r o j c d o u o f 1958,31 n w c n c r c s s i n 2 23 [2]. A l h o u g h h r w a s c o n i n u d d c r s i n c n c r d h f r o m 2 19 o 2 2 (b y 1.5 %), h r i s n r n s n d f o r f u r h r i m p r o v m e n o f d r u g s c r n i n g o o l s [2]. O u o f p p r o x i m a l y 15 d r u g s h m a k i o c l i n i c l r i l s i n h f i l d o f o n c o l o g y , o n l y o n w i l l u l i m a l y r c i v p p r o v l f r o m h f o o d O n d d r u g d m i n i s r i o n [3]. A s r s u l , i c o s s b o u U S \$ 2.8 b i l l i o n n d 15 y r s o n v r g o d i s c o v r n d d v l o p d r u g [4]. T h i s v i g o r o u s s c r n i n g p r o c s s h i g h l i g h s h n d f o r m o r f f i c i n d r u g s c r n i n g o o l s h p r c l i n i c l l v l .

C o n s q u n l y , h r h s b n n i n c r s d d o p i o n o f h r - d i m e n s i o n l i n v i r o m o d l s i n c n c r d r u g d v l o p m e n [5]. T h s m o d l s l l o w s i n g o n h u m a n c l l s w i h i n m o r r l i s i c h r - d i m e n s i o n l s r u c u r s . I h s b n s h o w n h b o h c o m p o s i o n n d

r c h i c u r p l y i m p o r n r o l s i n c l l u l r b h v i o r [5]. r o v r , i h s b n d r m i n d h h c h m o s n s i v i y o f c n c r c l l s i s f f c d b y b o h h s p c i f i c d r u g n d c l l l i n u s d [6]. F o r x m p l , P A N C - 1 p n e r i c c n e r c l l s c u l u r d i n f i b r o b l s d r i v d m a r i x s h o w d h i g h r r s i s n e o h d r u g p c l i x l c o m p r d o c l l s c u l u r d i n r d i o n l w o - d i m e n s i o n l c u l u r s . S i m i l r d r u g s n s i v i i s w e r o b s r v d b w e n w o - d i m e n s i o n l c u l u r s n d h o s c u l u r d i n h f i b r o b l s d r i v d m a r i x , w h n f o u r o h r d r u g s w e r s d o n h P A N C - 1 c l l s [7].

A n o h r s u d y o n h i n v i r o 3 D c l l c u l u r m o d l o f o r l c r e i n o g n s i s f o r r p i d s i n g o f c h m o h r p u i c g n s h s s u c c s s f u l l y d v l o p d 3 D p r c l i n i c l m o d l b y u s i n g s p h r o i d s [8]. H o w e r , c o m p r d o h 2 D m o d l s , h 3 D c l l c u l u r s b y s p h r o i d s w a r r - p o r d o c u s m a n y n i c n e r c o m p o u n d s o l o s f f i c y b c u s o f i n s u f f i c i n d r u g p n r i o n i n o h i n n r c o r o f h 3 D s r u c u r s [9]. H o w e r , o h r s u d i s h v d m o n s r d o p p o s i o b s r v i o n w h r 3 D c n c r s p h r o i d c u l u r i s m o r f f c i v f o r h d r u g u s d , i . . k i n s i n h i b i o r s [1]. I n d d i o n , h 3 D b i o p r i n d u m o r c l l c u l - 0 u r s w e r r p o r d o d m o n s r m o r r s i s n e o d r u g o x i c y 0

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have the 2D cell culture [11]. By better mimicking the *in vivo* environment using these three-dimensional models, more accurate information about toxicity and cellular responses can be obtained earlier in the drug development process.

Various methods for scaffold manufacturing, including three-dimensional bioprinting, are used to create intricate *in vitro* models [12–15]. Bioprinting deposits cells embedded in biomaterials, i.e. bioink layer by layer, enabling customizable and reproducible scaffolds that replicate complex *in vivo* structures and microenvironments [16, 17]. The bioinks, derived from natural and synthetic sources, must balance structural and physiological properties to support cell growth and maintain stability [18,19]. The bioink needs to support cell growth and development while still providing the dimensional stability required to mimic the structure of complex heterogeneous tissues. Key factors in selecting a bioink material include printability, shape fidelity, and support of cell viability and functionality [19]. Hydrogels made from natural polymers, such as alginate, cellulose, and gelatin are commonly used in bioprinting because of their ability to encapsulate cells, allow nutrient permeation, and mimic tissue stiffness [18,20–22].

The alginate, though shear-thinning, forms a soft gel and can limit construct height. Its printability improves when crosslinked with sodium-calcium ion exchange or blended with larger molecules, such as cellulose [19]. Cellulose, especially nanocellulose, offers promising mechanical properties and acts as a viscosity modifier in bioinks but lacks cell binding domains, affecting cellular attachment and proliferation [19]. On the other hand, gelatin exhibits favorable biocompatibility, biodegradability, and cell binding domains that encourage cellular adherence, differentiation, migration, and proliferation [18,19]. However, its poor printability and shape fidelity, along with a sol-gel transition under physiological conditions, pose challenges [19]. Combining alginate, cellulose, and gelatin allows for the tuning of both mechanical and physiological properties in bioprinted scaffolds.

Various types of small tissue models have already been developed to help in drug development, cancer research, and toxicology screenings [6,7,12]. By bioprinting cells into a three-dimensional structure, the efficiency of drug testing can be enhanced, potentially reducing costs and shortening the time drugs spend in preclinical trials [23]. Thus, the overall goal of this research was to develop a system for rapid and accurate drug testing of bioprinted cancer cell cultures. Specifically, this study focused on pancreatic cancer cells and assessed the effectiveness of various natural polymer hydrogels to achieve high printability and cell viability. These hydrogels were subsequently evaluated in conjunction with a spinning bioreactor, designed to serve as a drug testing platform that better replicates *in vivo* conditions.

2. Materials and methodology

2.1 Cell culture and bioink materials

Dulbecco's modified eagle media (DMEM), Seradigm fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from VWR (Sanborn, NY, USA). Antibiotic antimycotic solution was obtained from Corning (Manassas, VA, USA). Cell culture media was created by combined 89 % DMEM, 10 % FBS, and 1 % antibiotics. BxPC-3 pancreatic cancer cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Viability/cytotoxicity assay kits for animal live & dead cells were purchased from Biotium (Freemont, CA, USA). As bioink materials, cellulose nanocrystals (CNCs) were provided by the Forest Products Lab (FPL) (Madison, WI, USA) and CelluForce (CF) (Windsor, QC, CA). Carboxymethyl cellulose (CMC) sodium salt, medium viscosity, was ordered from MP Biomedicals (Solon, OH, USA). Gelatin was purchased from Zint (New Hampton, NY, USA), and alginic acid sodium salt, high viscosity powder was purchased from Alfa Aesar (Ward Hill, MA, USA). A 90 mM calcium chloride (CaCl_2) crosslinking

solution was created by dissolving CaCl_2 from Willpowder (Miami Beach, FL, USA) in deionized water.

2.2 Bioink formulations

To synthesize the bioinks, the desired concentration of cellulose or gelatin was first added to deionized water and mixed using magnetic stirring for 30 minutes or until a uniform solution was obtained. Then sodium alginate was slowly incorporated, and any aggregates were manually broken up until a uniform gel formed. The bioinks were sterilized at 121°C using the liquid setting of a BioClave 16 autoclave (Benchmark Scientific, Edison, NJ, USA) before cell culture use. The name and formulation of each bioink tested are displayed in Table 1.

2.3 3D printing process

Several parameters play critical roles in achieving consistent and uniform prints. These include print speed, line width, layer height, and fill pattern. Other parameters include physical variables of the printer, such as nozzle diameter, print temperature, and print pressure. Various combinations of the parameters were tested to achieve both smooth printability and good cell viability. The printer toolpath was created by converting a CAD file from Autodesk Inventor (San Rafael, CA, USA) to g-code using Slic3r software. A commercial bioink, Cellink Start, was used to print the structures, and optimize the parameters. Fig. 1 depicts the Cellink Inkredible + bioprinter (Gothenburg, Sweden) that was used to print the constructs as well as an example of the toolpath used to print a three-dimensional scaffold.

2.4 Analysis of cell viability in bio-printed scaffolds

To prepare the cells and bioink for printing, cell culture media containing BxPC-3 cells was mixed with bioink at a ratio of 1:10 resulting

Table 1
Summary of bioink names and formulations.

Bioink Name	Water (w/v%)	Gelatin (w/v%)	Alginate (w/v%)	Cellulose (w/v%)	Cellulose Provider
CMC-AI	94 c	0 c	2 c	4 c	MP Biomedicals
FPL-CNC-AI	94 c	0	2	4	Forest Product Lab
CF-CNC-AI	94 c	0 c	2	4 c	CelluForce
Gel-AI	93	5	2	0	N/A
Gel-AI-CNC	92.5	5	2	0.5	Forest Products Lab

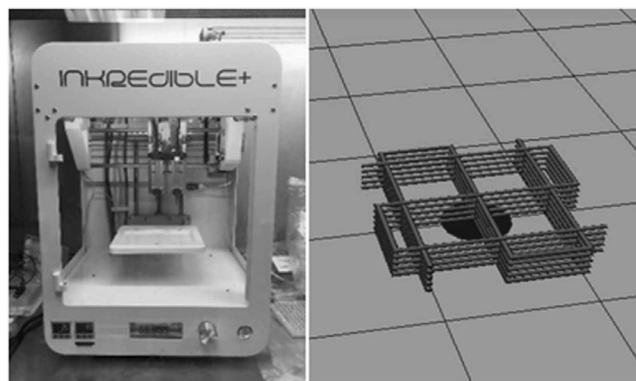


Fig. 1. Cellink Inkredible + bioprinter (left) and toolpath used to print a three-dimensional scaffold (right).

nal concentration of 1×10^6 viable cells/mL. Scaffolds were then printed directly into a 12 well plate using a Cellink Inkredible + extrusion bioprinter (Gothenburg, Sweden) with each scaffold requiring about 0.5 mL of bioink, resulting in a final cell density of approximately 5×10^5 viable cells/scaffold. The scaffolds were then crosslinked using a 90 mM CaCl₂ solution for 5 minutes. Following crosslinking, the scaffolds were washed twice with PBS and then incubated in cell culture media. The media on the scaffolds was refreshed every two days.

To determine cell viability in the scaffolds, a procedure outlined in a previous research was followed [24]. Briefly, a 4 μ M calcein AM/8 μ M EthD-III solution was prepared. The scaffolds were washed and incubated in DMEM without PBS for 30 minutes. Then the DMEM was removed, and the scaffolds were fully covered in the staining solution (2 mL per scaffold) and incubated for 1 hour. Next, the staining solution was removed, and the scaffolds were washed twice and incubated again in DMEM without PBS for 30 minutes. The scaffolds were transferred to a microscope slide and imaged by fluorescence microscopy (Leica DMI8, Wetzlar, Germany) using the FITC and Texas Red filters. Images at three different locations were analyzed for each scaffold. Cell viability was calculated from the resulting images using the Nation Institutes of Health (NIH) ImageJ software. Two scaffolds were tested for each time point, and the average was calculated. c

2.5 Rheology testing c

Rheology testing was performed using a TA Instruments AR-G2 rheometer (New Castle, DE, USA). All tests were performed at room temperature using 25-mm diameter parallel plate attachments and a 300- μ m gap. At least three replications were performed for each test, and the average was calculated. To study the viscosity of the samples, steady state flow tests were performed where the shear rate was varied from 0.1 to 500 s⁻¹. To determine the linear viscoelastic region (LVR) of the hydrogels, a strain sweep from 0.01 % to 100 % strain was performed at a frequency of 1 Hz. If the hydrogel did not leave the LVR in this strain range, the range was further increased for that bioink formulation. The end of the LVR was defined as the strain value where the complex modulus of the hydrogel decreased by 10 % from the constant plateau value [25]. Lastly, frequency sweeps from 0.01 to 10 Hz were conducted at strain values determined to be in the LVR from the strain sweeps. c

Theoretical calculations were used to compare the rheological results to the shearing that occurs in the nozzle during printing. The shear thinning properties of the bioinks were modeled using the power-law model, which is shown in Eq. (1). c

$$\eta = K \dot{\gamma}^{n-1} \quad (1)$$

where η is the apparent viscosity, K is the fluid consistency, $\dot{\gamma}$ is the shear rate, and n is the flow behavior index. Curve fitting was performed on the viscosity versus shear rate curves to calculate the values of K and n for different bioinks. To compare the viscosity of each bioink during printing, the shear rate in the nozzle was calculated. The Rabinowitsch correction was used to account for the non-Newtonian behavior of the bioinks, which is given by Eq. (2).

$$\dot{\gamma} = \frac{4Q}{\pi r^3} \left(\frac{3}{4} + \frac{1}{4n} \right) \quad (2)$$

where $\dot{\gamma}$ is the shear rate, Q is the volumetric flow rate, r is the radius of the nozzle, and n is the flow behavior index. The average shear rate at the tip of the nozzle was calculated by numerical integration of radii from 205 to 500 μ m using the measured flow rates of 13.5 and 9.9 μ L/s for bioinks CF-CNC-AL and CMC-AL respectively. Lastly, the average viscosity of the bioinks through the tip of the nozzle was calculated from Eq. 1 using the calculated shear rates. c

2.6 Bioink formulation c

Scaffolds of bioink CF-CNC-AL were constructed into molds and soaked in EpoDye from Struers Inc. (Cat. # 40300002, Cleveland, OH, USA). The prepared molds were visualized with a Zeiss LSM 700 laser scanning confocal microscope (Thornwood, NY, USA) using Plan-Apochromat 40x/1.3NA oil immersion lens and 20x/0.8NA objective lens where the 488/520 nm green channel was assigned for the dye. For 3D visualization, Z-stack images were taken up to 80 μ m deep into the mold structure. Computation of the Z-stack images was performed in Imaris (9.0.1) (Bitplane, South Windsor, CT, USA) software to obtain rendered reconstructions of the pores. The NIH ImageJ software was used to analyze the porosity and average pore diameter of the images.

2.7 Bioreactor design c

To simulate vasculature in the body, a spinning bioreactor was designed to provide media circulation in each of the wells. The structure of the spinning bioreactor was adapted from a previous study [26] where the mixers, plate cover, and top motor support were manufactured using a Formlabs Form 2 printer (Somerville, MA, USA) while the gears, bearings, fasteners, and electrical components were purchased from commercial vendors. The bioreactor was manufactured to fit over a standard 12 well plate, and the mixers were altered to create room for the scaffolds at the bottoms of the wells. Additionally, drug injection ports were created through the mixers to allow drug formulations to be injected into the individual wells using a micropipette. A speed controller was also added to allow for optimization of the media circulation rate. Fig. 2. shows the spinning bioreactor over a 12 well plate with the speed controller, and sample scaffolds printed into the 12 well plate.

In each bioreactor, a single motor powers a gear system that regulates the speed of the mixers placed in each separate well. Two spinning bioreactors were wired in parallel so that they could be controlled by a single speed controller. The speed controller was kept outside of the incubator to allow changes in speed without disrupting the contents of the incubator.

3. Results

3.1 Print parameter optimization

Print pressure has a significant impact on cell viability during the printing process because higher pressures result in higher shear stresses on the cells. Because of this, the largest standard nozzle size of 22 G was chosen. Additionally, it was found that smaller nozzles were more prone to clogging. From there, the print pressure was manipulated to determine what pressure was required to achieve uniform extrusion. It was found that a pressure of 25 kPa was adequate to achieve smooth flow out of the nozzle, and similar or higher pressures have been shown to produce high cell viabilities in other studies [27,28]. Next, the print speed was determined, which is affected by both nozzle diameter and print pressure. Too slow of a print speed can result in over-extrusion of material and poor print resolution, while too fast of a print speed can result in thinning and potentially breakage of the gel filament [29].

Line width and layer height mostly depend on the nozzle diameter. However, bioink rheological properties also play a role as some materials spread on the print bed due to gravity more than others. This spreading results in a lower layer height compared to the line width. For the nozzle size and material tested here (Cellink Start bioink), a layer height of 0.5 mm and a line width of 0.7 mm were selected. When lower layer heights or line widths were tested, over-extrusion and overlapping of material occurred. On the contrary, larger layer heights or line widths resulted in gaps between the material, and the gel filament had a greater tendency to break away from the printed structure, which frequently resulted in curling at the nozzle tip.



Fig. 2. Spinning bioreactor and speed controller (left), and scaffolds in a 12 well plate (right).

Lastly, different fill patterns including rectilinear and concentric were evaluated to see what would provide the best print resolution and shape fidelity. It was determined that rectilinear was more effective for print geometries with many straight lines or sharp corners while concentric was a better choice for geometries with more curved or round features. Since the scaffolds used for this application were rectangular with mostly straight lines, the rectilinear fill pattern was chosen. These parameters were then verified by printing a variety of geometries including a cube, a hollow cylinder, and a pancreas model. Table 2 displays the final print settings.

3.2 Printability and shape fidelity of bioink formulations

Scaffolds of each bioink were 3D-printed to assess the printability and adherence to intended shape. Fig. 3. offers a side-by-side comparison of the scaffolds produced with each bioink. Bioink CMC-Al exhibited poor print resolution, as multiple-layered scaffold would flow together once deposited on the print bed. In contrast, bioink FPL-CNC-Al, containing CNCs from the FPL instead of CMC, demonstrated superior print quality. It allowed for the creation of multiple layers while still maintaining macroscopic pores essential for nutrient and waste diffusion. Additionally, bioink FPL-CNC-Al required a lower pressure (25 kPa) for printing compared to bioink CMC-Al (50kPa). This difference can be attributed to the better shear thinning properties of CNCs com-

pared to CMC. To compare different CNCs, bioinks CF-CNC-Al and FPL-CNC-Al were assessed to determine the effects of the CNC processing technique on bioink properties. Regarding printability, both bioinks exhibited highly similar properties and were printed at 25 kPa. Bioink CMC-Al was eliminated from potential applications in drug testing due to its tendency to flow after deposition on the print bed.

In testing the printability of gelatin as the major bioink component (bioink Gel-Al) as opposed to cellulose, bioink Gel-Al displayed a stable structure with minimal spreading on the print bed. However, the bioink did not flow smoothly out of the nozzle, resulting in a more uneven print geometry, and necessitated a pressure of 65 kPa. To improve its printability, CNCs were introduced to create bioink Gel-Al-CNC. This led to a smoother and more uniform print and slightly reduced the required pressure to 60 kPa. Nevertheless, when scaffolds of either bioink Gel-CNC-Al or Gel-Al were cultured in an incubator for several days, the gelatin dissolved from the scaffolds, leading to an unstable structure. Additionally, these bioinks encountered frequent contamination issues; autoclave sterilization degraded the gelatin, and ultraviolet sterilization was ineffective. Because of these challenges, both bioink Gel-Al and bioink Gel-Al-CNC were excluded from consideration for future drug testing applications. Consequently, bioinks FPL-CNC-Al and CF-CNC-Al were selected for further testing, as they offered superior printability, better stability during incubation, and fewer contamination issues.

3.3 Rheology testing

Rheology testing was performed to analyze the response of each bioink constituent to shear forces, which are also experienced during extrusion through the print nozzle. Using steady state flow testing, the viscosities of bioink CF-CNC-Al and its individual components were compared to bioink CMC-Al and its components to better understand the difference in printability between the bioinks. In Fig. 4(a), it can be seen that all of the components and bioinks showed shear thinning behavior, represented by a decrease in viscosity over a large range of shear rates with no apparent plateaus. This is caused by the alignment

Table 2
Summary of final printer process parameters.

Slic3r Parameters	
Print Speed	5 mm/s
Line Width	0.7 mm
Layer Height	0.5 mm
Fill Pattern	Rectilinear
Printer Parameters	
Nozzle Diameter	22 G (410 μ m)
Print Temperature	Room Temperature
Print Pressure	25 kPa

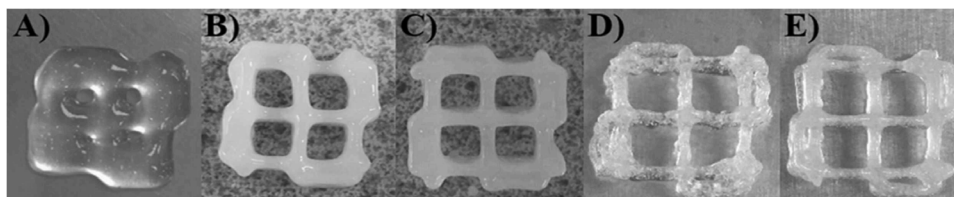


Fig. 3. Scaffolds printed with bioinks A) CMC-Al, B) FPL-CNC-Al, C) CF-CNC-Al, D) Gel-Al, and E) Gel-Al-CNC.

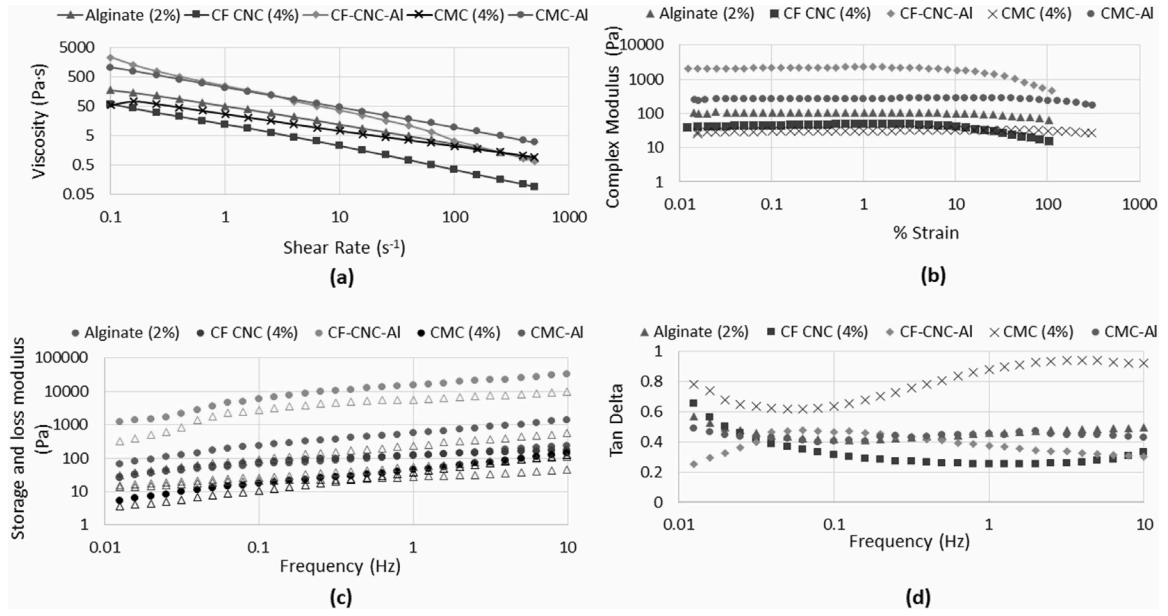


Fig. 4. (a) Viscosities of various solutions and bioinks during steady state flow testing; (b) complex modulus of various solutions and bioinks during strain sweep testing; (c) Storage modulus (closed circles) and loss modulus (open triangles) and (d) tan delta values for various solutions and bioinks during frequency sweep testing.

of polymer chains that occurs when a shear force is applied. Compared to the CMC solution, the CNC solution showed better shear thinning properties as its initial viscosity was slightly higher, and it exhibited a steeper decrease in viscosity as the shear rate increased. It should be noted as well that the 2 % alginate solution showed a higher viscosity than the 4 % CNC solution, indicating that alginate had a more substantial effect on the bioink's overall viscosity than the CNCs did.

When comparing bioinks CF-CNC-Al and CMC-Al, the differences in shear thinning can help to explain the reason that bioink CF-CNC-Al showed better printability and can be printed at a lower pressure. Bioinks CF-CNC-Al and CMC-Al showed viscosities of 2314 and 1046 Pa·s respectively at a shear rate of 0.1 s^{-1} . At low shear rates like this, a higher viscosity is more desirable so that printed constructs will remain stable and resist spreading on the print bed. Conversely, at higher shear rates, such as when the bioinks are being forced through the print nozzle, lower viscosities are more desirable because they allow for smoother flow at lower pressures. From the theoretical calculations, it was determined that bioink CF-CNC-Al has a lower viscosity when it is extruded through the nozzle. Table 3 displays the results of the theoretical calculations for shearing through the print nozzle. The combination of higher viscosity at rest and lower viscosity at the higher shear rate indicates that bioink CF-CNC-Al is better suited for extrusion bioprinting.

Strain sweeps were performed to determine the LVR for each solution, and the results are displayed in Fig. 4(b). The CNC solution showed a sharp decrease in complex modulus as it exited the LVR, indicating a breakage of the gel into larger pieces. Conversely, the CMC solution showed a more gradual decrease in complex modulus once it exited the LVR, suggesting a more gradual and homogeneous breakdown

of the gel structure. Similar trends are visible for bioinks CF-CNC-Al and CMC-Al respectively.

The Table 4 shows the end of the LVR and the overall strain range that was tested for each solution and bioink. The alginate solution, CF CNC solution, and bioink CF-CNC-Al exhibited similar LVRs, while the CMC solution and bioink CMC-Al were stable at much higher strain values. Based on the strain sweeps, 1 % strain was chosen for the frequency sweeps because this strain percentage clearly fell within the plateau region of the strain sweep for each solution.

Frequency sweeps for each of the solutions at a constant strain of 1 % are displayed in Fig. 4(c). For each case, both the storage and loss moduli of the solutions increased over the duration of the test. Bioink CF-CNC-Al consistently exhibited the highest storage modulus throughout the frequency sweep, which indicates its higher stiffness or gel strength.

The storage modulus of each solution was greater than the loss modulus over the range of frequencies tested. This overall higher storage modulus indicates that the materials exhibit more elastic than viscous behavior, which is also reflected in the tan delta values of less than one, as seen in Fig. 4(d). However, for the CMC solution, the tan delta value was closer to one, indicating that the material displayed nearly equal elastic and viscous behavior, and that it exhibited more viscous behavior when compared to the other solutions.

3.4 Cell viability

Bioinks FPL-CNC-Al and CF-CNC-Al were tested to determine which better supported cell viability and proliferation of BxPC-3 cells. Fig. 5 (a). shows a comparison of the resulting cell viabilities for each bioink,

Table 3
Analysis of shear rate and viscosity in the nozzle during printing.

Bioink	Flow Behavior Index (n)	Fluid Consistency (K)	Nozzle Shear Rate (s^{-1})	Viscosity (Pa·s)
CF-CNC-Al	0.078	257	2350	0.20
CMC-Al	0.315	222	675	2.57

Table 4
Strain sweep results for various solutions and bioink formulations.

Solution	Alginate (2 %)	CF CNCs (4 %)	CF-CNC-Al	CMC (4 %)	CMC-Al
Strain Range Tested	0.01–100 %	0.01–100 %	0.01–100 %	0.01–300 %	0.01–300 %
End of LVR	16.01 %	7.52 %	7.52 %	236.33 %	75.46 %

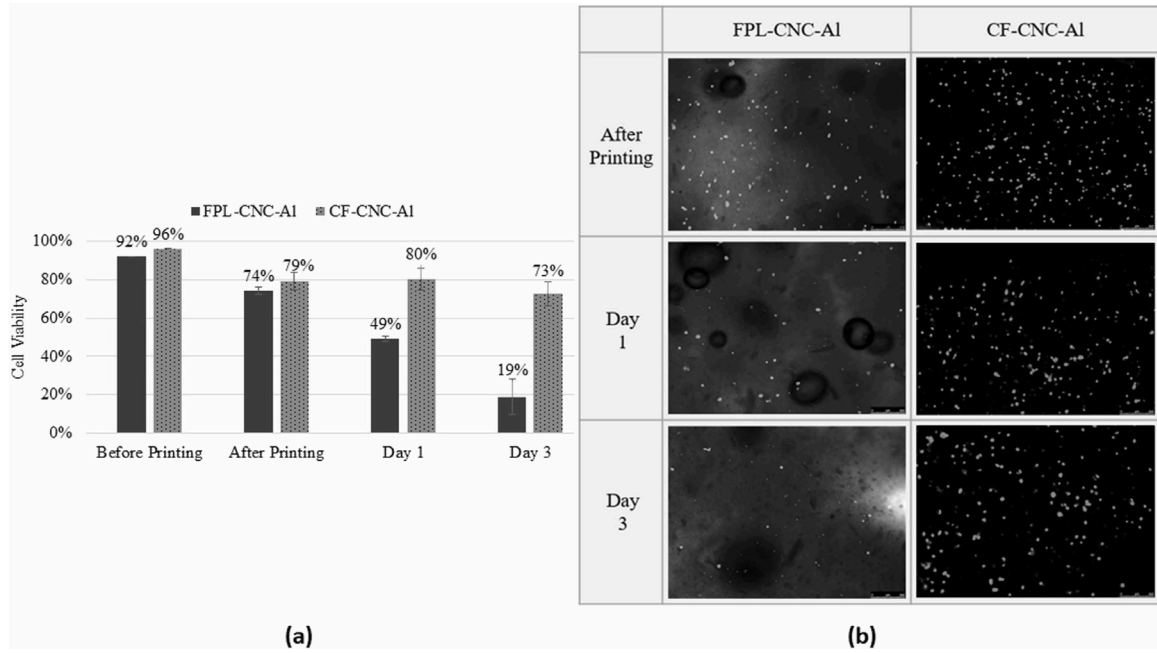


Fig. 5. (a) Cell viability and (b) fluorescence microscopy images of BxPC-3 cells in bioinks FPL-CNC-AI and CF-CNC-AI.

and Fig. 3 shows the corresponding fluorescence microscopy images. Cell viability after printing in bioink FPL-CNC-AI was 74 % and in bioink CF-CNC-AI was 79 %. The initial decrease in cell viability after the printing process can be attributed to the handling and shear stresses that the cells experience during the preparation and printing processes. Additionally, the crosslinking process can have negative effects on cell viability because the cells are exposed to high calcium concentrations [30]. Therefore, care should be taken to minimize the crosslinking time and concentration. When culturing the scaffolds for multiple days, the bioink FPL-CNC-AI showed a rapid decrease in cell viability compared to the bioink CF-CNC-AI. After three days of culture, viable cells were sparse throughout the matrix of the bioink FPL-CNC-AI, whereas in bioink CF-CNC-AI, the distribution and number of viable cells were similar to after printing, as shown in Fig. 5(b).

Since both bioinks contained both CNCs and alginate, it was hypothesized that the differences in cell viability could be attributed to the production method used to manufacture the CNCs. Both the FPL and CF manufacture their CNCs using sulfuric acid hydrolysis [31]. Therefore, the pH of 4 % CNC solutions from each manufacturer were measured to determine the degree of neutralization achieved by the acid in the production process. The pH of the FPL CNC solution was 5.62, whereas the pH for the CF CNC solution was 7.32. Therefore, it can be concluded that the poor cell viability in bioink FPL-CNC-AI can be attributed to the acidity of the CNCs. Other studies have verified that changes in pH can affect cell viability, as primary keratinocyte and fibroblast cultures showed complete cell death after one day in culture conditions at pH 5.5 [32]. Based on these results, bioink CF-CNC-AI was chosen for future testing to better evaluate its properties and to test it in conjunction with the spinning bioreactor.

3.5 Confocal microscopy

To analyze the porous structure of bioink CF-CNC-AI in its hydrated state, confocal microscopy was performed. Fig. 6. shows the resulting microscopy images of the bioink. The pores in the hydrogel were stained green by the dye while the solid material remained black. The cylindrical and oval pores appear to have uniform distribution throughout the hydrogel; however, the shape of the pores may be altered by the

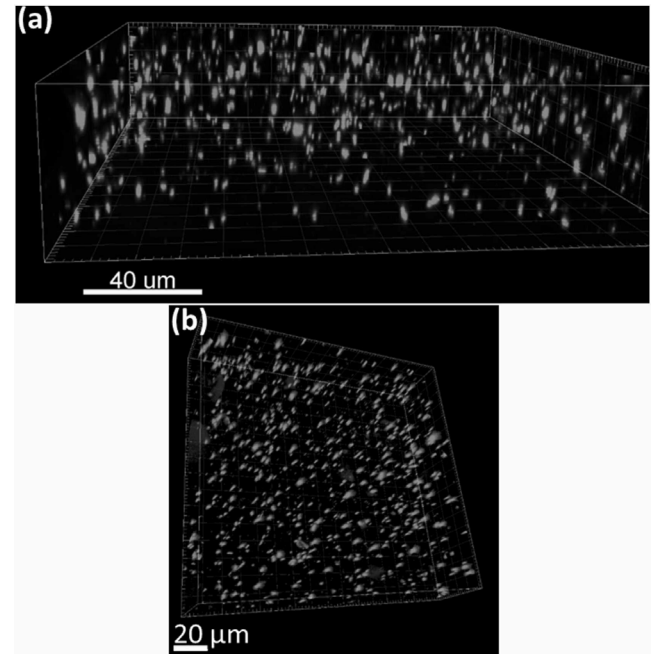


Fig. 6. Confocal microscopy image of bioink CF-CNC-AI at 40x magnification showing (a) a lateral view and (b) a top view of the pores.

vertical reconstruction that occurs during image processing of the Z-stack images. When analyzing the well stained areas, the hydrogel was found to have an average pore diameter of $12.00 \pm 4.48 \mu\text{m}$ and a porosity of $20.81 \pm 2.73 \%$. These pores provide space for nutrients and waste to move in and out of the deeper parts of the scaffold while also providing space for cells to grow and proliferate.

Cell viability assays were performed on scaffolds of bioink CF-CNC-AL with and without the use of the spinning bioreactor to evaluate its effectiveness in the system. Fig. 7. displays the resulting cell viabilities. No significant differences were observed in cell viability after printing or over the first two days of culture, as cell viability remained stable between 70 % and 80 %. However, on the third and fourth days, the scaffolds that were tested with the spinning bioreactor showed a dramatic decrease in cell viability compared to the control scaffolds. This was likely caused by the mixers disturbing the cell cultures, as it was observed that some of the mixers were causing the scaffolds to move in the wells. Therefore, future considerations should be taken to ensure that the clearance between the mixers and scaffolds is adequate and that the mixing speed is not too high. c

Fig. 8. displays the fluorescence microscopy images of the scaffolds, illustrating the comparison between the scaffold conditions with and without the use of the spinning bioreactor. These images depict the occurrence of cell death on days three and four in the presence of the spinning bioreactor. Additionally, it is noteworthy that throughout the experiment conducted without the spinning bioreactor, the size of the viable cells seems to have increased. This could be in part due to the formation of cellular spheroids. When comparing the average diameter of the green dots after printing with that on day four, an increase in size from 21.18 ± 7.06 – $33.40 \pm 10.67 \mu\text{m}$ was observed. This is relevant to the cell viability assays as each green dot was assumed to represent a singular cell by the NIH ImageJ software. Therefore, if small cellular spheroids are actually forming, a different assay would provide more accurate cell viability data. For example, an MTT assay measures cellular metabolic activity instead of counting individual cells, and it has been used with three-dimensional cultures in other studies [33]. It would be useful in comparing the effectiveness of different drug formulations against each other. Lastly, since tumors have a dense structure with necrotic cores, the development of cellular spheroids should produce drug testing results that translate well from this in vitro model to in vivo. c

4. Discussion c

Crafting a tissue surrogate that accurately replicates the distinct environment of an organ stands as a pivotal element in drug development. The precision of the model in mimicking the pancreatic niche directly correlates with its efficacy in predicting drug responses. Over the past decade, researchers have dedicated several efforts to engineer in vitro models specifically designed to mimic the characteristics of the pancreas. The pressing demand is to harness the growing potential and

complexity of innovative 3D in vitro cell models within tissue-mimicking environments. In recent times, 3D bioprinting has emerged as a promising technology for constructing tissue-based platforms with high reproducibility and scalability. A critical aspect in developing a pharmaceutically valuable 3D bioprint model lies in selecting a printable biomaterial, commonly known as bioink. This bioink plays a pivotal role in determining cancer cell phenotypes and the biophysical properties of the tissue. This paper delves into the development of bioinks tailored for establishing a pancreatic cancer model and investigating the cell viability of the resulting bioinks. The focus is on exploring the cellular and rheological aspects of the bioinks to advance understanding of their application in pharmaceutical contexts.

All bioinks investigated in this study displayed a non-Newtonian, shear-thinning behavior characterized by pseudo-elasticity. Notably, CF-CNC-AL exhibited higher viscosity at lower shear rates compared to others, while demonstrating lower viscosity at higher shear rates than CMC-AL. The heightened static viscosity of CF-CNC-AL resulted in minimal spreading, and its low viscosity during printing facilitated extrusion. Beyond shear-thinning, CF-CNC-AL likely exhibited low hysteresis, allowing for the immediate recovery of high static viscosity post-ink extrusion. This property ensures shape fidelity and prevents the collapse of intricate 3D bioprinted structures, aligning with the characteristics of ideal bioinks as observed in previous studies [34]. The observed increase in shear-thinning in CNC-containing inks is attributed to alterations in CNC–CNC and CNC–polymer interactions under shear stress [34,35]. In the static state, alginate molecules in CF-CNC-AL form electrostatic interactions with the negatively charged CNC surfaces, wrapping around the CNCs [35,36]. When shear stress is applied, both the CNCs at the solution–solid interface and the alginate wrapped around CNCs align with the shear stress direction [35,36]. This alignment leads to the slipping of CNCs past each other, causing tangled polymers to realign and a significant decrease in viscosity. Compared to hybrid bioinks documented in the literature [35,37], the CF-CNC-AL bioink in this study exhibited more pronounced shear-thinning behavior and up to an order of magnitude higher viscosity in the static state. These findings underscore the superior printing capabilities of the CF-CNC-AL bioink.

CF-CNC-AL demonstrated a higher storage modulus value than other scaffolds, signifying the positive impact of incorporated CNCs on the mechanical strength of the printed scaffolds. This improvement in mechanical strength is ascribed to the formation of a more interconnected polymeric network structure, which restricts the motion of polymer chains and consequently elevates the storage modulus [38,39]. Furthermore, an increase in loss modulus was observed in the CF-CNC-AL scaffolds compared to other scaffolds across the measurement range. This observation also points to the development of a highly interconnected c

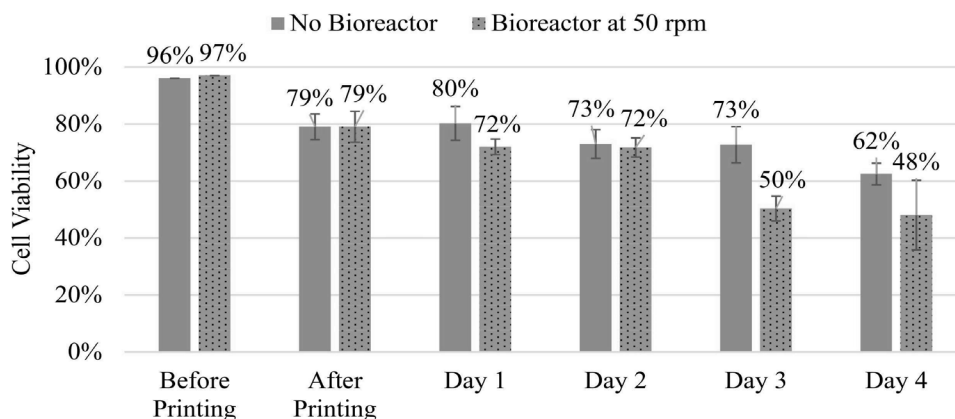


Fig. 7. Cell viability of BxPC-3 cells in bioink CF-CNC-AL with and without the use of the spinning bioreactor. c

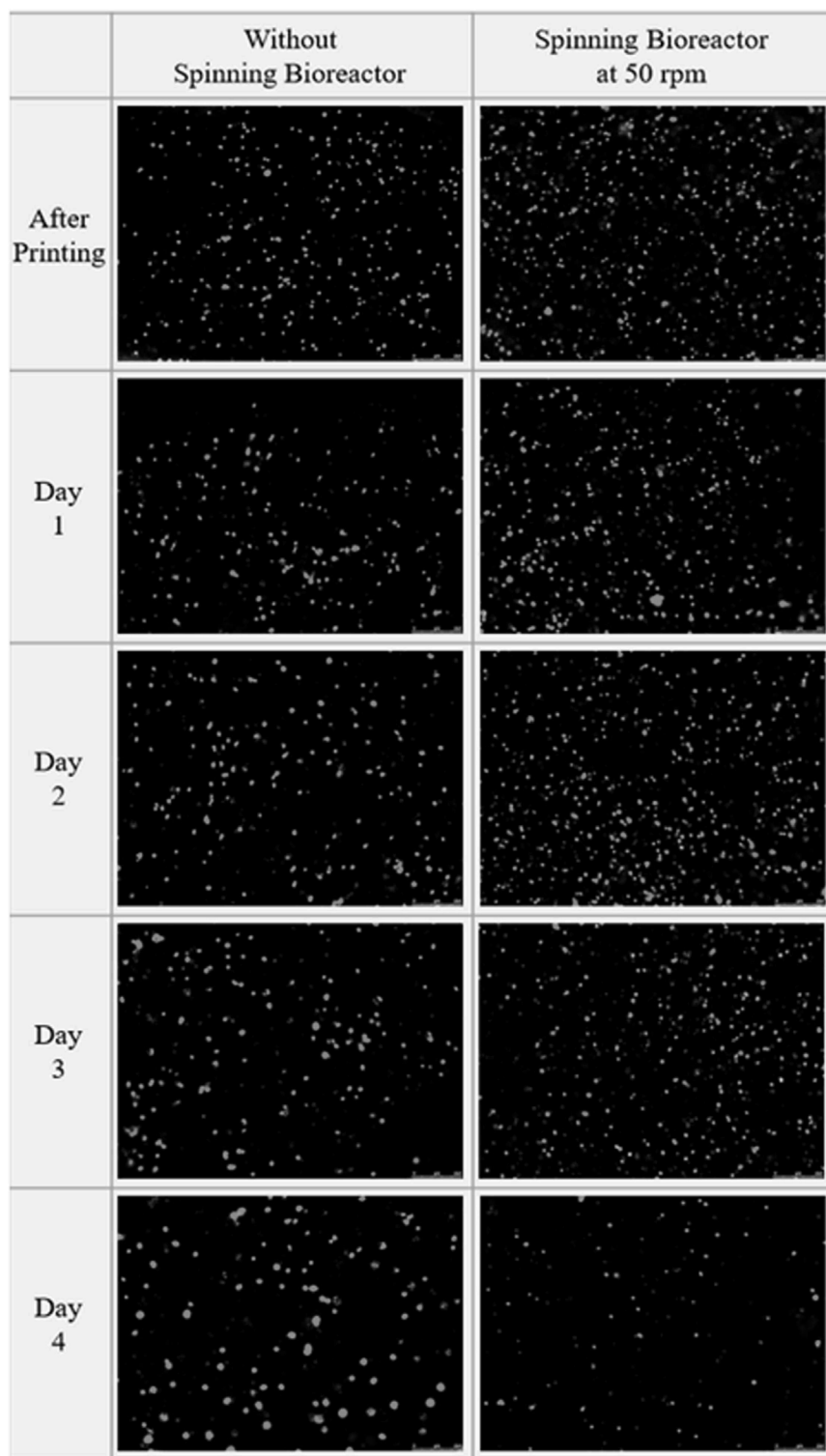


Fig. 8. Fluorescence microscopy images of BxPC-3 cells in bioinks CF-CNC-Al with and without the use of the spinning bioreactor.

polymeric network structure in CF-CNC-Al. Notably, the loss modulus values remained lower than the storage modulus values throughout the frequency range, indicating that CF-CNC-Al exhibits more solid-like behavior. Consequently, CF-CNC-Al produced scaffolds with excellent structural fidelity.

In this study, CF-CNC-Al exhibited higher cell viability compared to other bioinks, attributed to the lower acidity in the CF-CNC solution.

The porous structures achieved through 3D printing facilitated superior oxygen, nutrient, and waste transport when compared to bulk scaffolds produced by non-printed methods [40]. In contrast to a similar study using a formulation with 2 % alginate and 4 % CNC in the bioink [37], the current CF-CNC-Al bioink demonstrated enhanced cell viability even after a 3-day culture period. Nonetheless, there was a decline in cell viability post-printing and after 3 days of culture. Various factors c

may contribute to this reduced cell viability in CNC-alginate bioink. Datta *et al* proposed that the bioprinting process itself, particularly the mechanical stresses during extrusion, can induce cell death [41]. This assertion finds support in the work of Kong *et al*, which indicated that the high viscosity of pre-gelled solutions may decrease cell viability due to the elevated shear forces required to mix cells with such solutions [42].

Furthermore, the concentration of CNCs and their surface charge play a crucial role in influencing cell viability in the bioinks. Dutta *et al* reported that optimal cell viability was maintained in CNC/Alg/Gel bioinks up to a 1 % CNC concentration, beyond which cell survival started to decline [39]. Additionally, both Mahmoud *et al* and Nguyen *et al* emphasized the significance of surface charge of CNCs in cellular uptake and cytotoxicity [43,44]. Positively charged CNCs exhibited higher uptake and lower cytotoxicity. In the CF-CNC-Al bioink used in this study, the combination of high solution viscosity, a 4 % CNC concentration, and a negatively charged CNC surface may have collectively contributed to the decreasing cell viability over time.

Moreover, the concentration of alginate plays a vital role in terms of cell viability in cell-laden scaffolds. Previous studies indicated that higher alginate concentration composite scaffolds had increased stiffness and lower nutrition diffusion rates, causing more damage compared to lower concentration scaffolds [40,45]. Park *et al* demonstrated that low alginate density may enable better mass transfer, cell migration, and growth in softer environments compared to bioinks containing high alginate density [46]. Additionally, it was reported that cells displayed a spherical morphology in scaffolds with higher alginate concentrations, while lower alginate concentrations led to a spreading morphology, forming a complex interconnected network between cells [47].

In this study, the spheroids in CF-CNC-Al scaffolds with 2 % alginate were more similar to the spherical morphology of cells in scaffolds with higher alginate concentration, which agrees with the literature for same concentration of alginate [45]. The stiffness of the CF-CNC-Al was also high, suggesting that the increased crosslinking between matrix macromolecules in the high stiffness group might create a matrix that is more challenging to degrade, explaining the lower proportion of spreading cells and, consequently, impacting cell viability. For instance, Gonzalez-Fernandez *et al* reported that pre-crosslinked alginate bioinks led to lower cell viability [48]. Therefore, the high alginate concentration and its pre-crosslinking in the CF-CNC-Al bioink resulted in high stiffness and low cell viability.

Besides, a rotating bioreactor was designed to provide medium circulation to simulate the vascular system in vivo as well. The results showed that the scaffolds with the use of a bioreactor had increasingly less cell viability than the control group without the use of a bioreactor. The reason for this decreasing cell viability was the reactor speed, which was 50 rpm. However, the optimized reactor speed was reported to be less than 20 rpm to avoid disruption in cell attachment [49,50]. In future studies, the pumping system of the bioreactor can be engineered by controlling multiple parameters to recreate human physiological flow rate, shear stress, and pressure and investigate the effects of fluid dynamics on vascular tissue engineering. Besides, a parameter study to find the optimal concentration and surface charge properties of alginate and CNCs is required to maintain the simultaneous structural fidelity and cell viability of cell-laden scaffolds.

5. Conclusions

This study compares the effectiveness of various natural polymer hydrogels for use in a platform for pancreatic cancer drug testing. These natural polymer hydrogels replicate the soft tissue environment of the pancreas, while a spinning bioreactor simulates the vasculature around the artificial tumor environment. It was demonstrated that the CNC and alginate hydrogel exhibited better printability and shape fidelity than a

CMC and alginate hydrogel. This observation was confirmed through both rheological testing and theoretical calculations. Specifically, the CNC and alginate hydrogel exhibited increased viscosity at low shear rates, at a condition near to the rest. Conversely, the viscosity of the bioink decreased when subjected to the nozzle shear rate. Additionally, the CNC and alginate hydrogel displayed better stability under physiological conditions than the gelatin and alginate hydrogel, which degraded over the course of multiple days in the incubator. Cell viability in the CNC and alginate hydrogel decreased from around 80–60 % over four days of culture. It was hypothesized that the spinning bioreactor would help to improve the cell viability over time. However, it disturbed the cell cultures, which negatively affected the cell viability. Nevertheless, this system shows potential for pancreatic cancer drug testing, as it replicates the three-dimensional soft tissue structure as well as the surrounding vascularization.

Uncited reference

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CRediT authorship contribution statement

Ismat Ara: Writing – review & editing, Writing – original draft. **Jessica E Pullan:** Investigation, Data curation. **Sanku Mallik:** Methodology, Conceptualization. **Dilpreet Bajwa:** Writing – review & editing, Supervision. **Chad Rehovsky:** Writing – original draft, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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