Upgrading Hepatic Differentiation and Functions on 3D Printed Silk–Decellularized Liver Hybrid Scaffolds

Aarushi Sharma, # Preety Rawal, # Dinesh M Tripathi, Dashrath Alodiya, Shiv K Sarin, Savneet Kaur,* and Sourabh Ghosh*

Cite This: ACS Biomater. Sci. Eng. 2021, 7, 3861−3873

ABSTRACT: We developed hybrid liver-specific three-dimensional (3D) printed scaffolds using a solubilized native decellularized liver (DCL) matrix and silk fibroin (SF) and investigated their ability to support functional cultures of hepatic cells. Rat livers were decellularized by perfusing detergents via the portal vein, solubilized using pepsin to form DCL, and characterized. SF blended with gelatin (8% w/v) was optimized with varying percentages of DCL to obtain silk gelatin−DCL bioink (SG−DCL). Different compositions of SG−DCL were studied by rheology for optimum versatility and print fidelity. 3D printed six-layered scaffolds were fabricated using a sophisticated direct-write 3D bioprinter. Huh7 cells were cultured on the 3D printed scaffolds for 3 weeks. 3D printed SG scaffolds without DCL along with 2D films (SG and SG−DCL) and 2D culture on tissue culture Petri dish control were used for comparative studies. The DCL matrix showed the absence of cells in histology and SEM. The combined SG−DCL ink at all of the studied DCL percentages (1−10%) revealed shear-thinning behavior in the printable range. The storage modulus value for the SG−DCL ink at all DCL percentages was higher than the loss modulus. In comparison to 2D controls, hepatic cells cultured on 3D SG−DCL revealed increased proliferation until 2 weeks and an upregulated expression of hepatocyte markers, including asialoglycoprotein receptor 1 (ASGR1). The Wnt pathway gene β-catenin was upregulated by more than 4-fold in 3D SG−DCL on day 3, while it showed a decline on day 7 as compared to 3D SG and also 2D controls. The expression of the epithelial cell adhesion molecule (EpCAM) was however lower in both 2D SG−DCL (2-fold) and 3D SG−DCL (2.5-fold) as compared to that in 2D controls. Immunofluorescence studies validated the protein expression of ASGR1 in 3D SG−DCL. Albumin (ALB) was not identified on SG scaffolds but prominently expressed in 3D SG−DCL constructs. In comparison to 2D SG, both ALB (1.8-fold) and urea (5-fold) were enhanced in cells cultured on 3D SG−DCL on day 7 of culture. Hence, the SG−DCL 3D printed scaffolds provide a conductive microenvironment for elevating differentiation and functions of hepatic cells possibly through an involvement of the Wnt/β-catenin signaling pathway.

KEYWORDS: silk, decellularized matrix, liver, 3D printed scaffold

1. INTRODUCTION

Liver has exceptional regenerative capability. However, long-term chronic liver diseases, caused as a result of toxicity, viral and inflammatory responses, and cancer, often lead to liver failure, which is life-threatening in many cases.1 The most common treatment strategy available in such scenario is liver transplantation; however, the issues related to donor and recipient immunological mismatch and acute shortage of suitable donor organs restrict the applicability of this process.2,3 Thus, there is need for an alternative strategy for translational applications that can be used instead of liver transplantation or a strategy that can be used for improved understanding of the pathophysiology of liver diseases, drug screening, detoxification, etc.

After allograft hepatic transplantation, the next best option is cellular-level transplantation using hepatocytes or progenitor cells.3 However, in vitro culture of primary hepatocytes is challenging as they are short-lived in culture. Furthermore, rapid loss in the proliferation rate of hepatocytes has been observed 3−5 days postisolation due to their tendency to undergo dedifferentiation, the loss of characteristic hepatocyte morphology and functionality.4−6 Different biomaterial scaffolds and culture strategies are being tested to optimize hepatocyte cultures in vitro. For example, decellularized liver (DCL) tissue and hydrogels based on DCL extracellular matrix (ECM) have been used extensively for liver tissue engineering, where all of the cellular and immunogenic components of
ECM proteins are removed only to retain the native ECM. Recently, a few studies have reported three-dimensional (3D) cultures of hepatocytes along with other liver cells, including endothelial and stellate cells, to form a functional liver construct. Minami et al. developed a 3D liver tissue equivalent using human-induced pluripotent stem cells and rat DCL-based scaffolds that expressed albumin (ALB) and cytochrome P450 3A4 (CYP3A4) genes after 48 h of recellularization. Mazza et al. cultured human cell line hepatic stellate cells, hepatoblastoma (HepG2) cells, and hepatocellular carcinoma (Sk-Hep-1) cells on human DCL scaffolds for 21 days that showed decent proliferation and ECM remodeling. It is important to mention here that, while use of DCL matrix seems like a promising approach for liver regeneration, native DCL scaffolds do not exhibit optimum mechanical and biological properties to support long-term viability and functionality of hepatocytes. Therefore, combining an optimum biomaterial with decellularized ECM could probably help to improve the biological functionality of the bioengineered liver constructs.

Furthermore, conventional approaches of polymeric 3D porous scaffold-based liver tissue engineering do not lead to recapitulation of precise organization of the liver cells. The traditional 3D porous scaffold-based culture system used for expansion and culture of hepatocytes fails to recapitulate the complex native architecture of liver tissue. Additionally, a lack of reciprocal interactions related to hepatocyte–hepatocyte and hepatocyte–ECM interactions in these traditional culture systems causes a quick loss of bioactivities of the hepatocyte. Hence, advanced biofabrication techniques of tissue engineering are requisite to enable the precise engineering of complex tissue structures including the liver. In the last years, a few studies have also demonstrated the potential of 3D printing techniques in printing hepatic tissues. However, a big challenge that hinders the 3D printing technique is an appropriate bioink. An ideal bioink should have desired biophysical and biological properties to promote the growth of cells and, at the same time, maintain their functions.

In our previous studies, we have extensively used silk fibroin (SF) hydrogel and gelatin-based bioink for the development of cartilage, bone, and skin tissue equivalents. For the first time, we reported that silk-gelatin (namely, SG) bioink can regulate several molecular signaling pathways like Wnt/β-catenin, Indian hedgehog, and bone morphogenetic protein signaling pathways. These findings provide strong justification of using SF protein-based bioink for liver regeneration, as Wnt/β-catenin has been observed to play a significant role in liver tissue development and formation of a mature liver organ.

In this study, we hypothesized that (1) DCL ECM would serve as an excellent biomimetic bioink as it would provide liver-specific regenerative signals to the implanted cells, (2) combining silk with the DCL would help to improve the biophysical properties and printing fidelity of the DCL bioink, and (3) the 3D printed constructs developed using the SG–DCL ink would further help in simulating liver tissue-specific gene and protein expression during in vitro culture of hepatocytes. Therefore, in the current study, we blended the SF-based hydrogel with DCL ECM to develop a liver-specific bioink, fabricated and characterized 3D printed scaffolds using this bioink, and also investigated their potential to support the culture of hepatocytes in vitro. Further, we assessed their gene and protein expression and release of ALB and urea on these SG–DCL constructs as compared to only SG constructs.

2. MATERIALS AND METHODS

2.1. Preparation of SF Solution. SF was isolated from Bombyx mori silk cocoons as mentioned in our previous study. Briefly, 5 g of silk cocoons was cut into small pieces and treated with 0.02 M Na2CO3 twice for 30 min at 100 °C. The silk fibers thus obtained were washed with deionized water and air-dried. SF fibers were then dissolved in 9.3 M LiBr solution at 60 °C for 4 h. The dissolved SF solution was dialyzed against deionized water for 48 h and stored at 4 °C.

2.2. Preparation and Characterization of DCL ECM. For anesthesia, Sprague–Dawley rats were given intraperitoneal injections of a cocktail of ketamine@75–100 mg/kg body weight + xylazine@10–12 mg/kg body weight. In the anesthetized animals, a large U-shaped cut was made on the abdomen to expose the peritoneal cavity. The portal vein was exposed by two cotton swabs to gently move the abdominal viscera to the right side. Thereafter, two sterile 2.0 silk sutures were tied underneath the portal vein to cannulate the portal vein with a 6–18 gauge intravenous catheter and the catheter was secured in place by tightening the loosely tied sutures from the previous step. First, about 1–2 mL of heparin sodium (100 U/mL) was injected through the vein for anticoagulation. Then, the infrainferior vena cava was transected to allow the outflow of the perfusate. Phosphate-buffered saline (PBS, 50 mL) was perfused through the portal vein to clear blood from the liver.

![Figure 1](https://doi.org/10.1021/acsbiomaterials.1c00671)

**Figure 1.** Decellularization, solubilization, and characterization of the decellularized rat liver. (a) Portal vein cannulation for perfusion of 1% Triton-X 100 and 0.5% SDS, with 0.1% ammonium hydroxide. (b) Partially DCL after 12 h of processing. (c) Completely DCL after 24 h. (d) Dissolved liver tissue prepared in pepsin solution. (e) Histological (H&E) staining of the DCL showing the presence of vascular network. (f) DAPI staining of the DCL matrix showing the absence of nuclear materials.

Then, the suprahepatic inferior vena cava, the hepatic artery, and the common bile duct were freed. The whole liver was isolated and transferred to a cell culture dish. The cannulae in the portal veins were attached to a pump (Masterflex L/S peristaltic pump with a Masterflex L/S easy load pump head and L/S 16G tubing, Cole-Palmer Instrument Co., Vernon Hills, IL, USA), and distilled water was perfused through the portal vein at a rate of approximately 5 mL/min (rat and ferret livers). Subsequently, 1% Triton-X 100 and 0.5% sodium dodecyl sulfate with 0.1% ammonium hydroxide (Sigma-Aldrich) were perfused through the livers to decellularize the organ for about 20–24 h (Figure 1b). Finally, the liver was perfused with PBS for 4 h to wash out the decellularization detergent (Figure 1c). The DCL was stored at −80 °C for the lyophilization process. The sample was processed for lyophilization for 48 h, and then, it was removed from the lyophilizer and kept in an airtight container. Then,
the sample was dipped in liquid nitrogen and crushed to a powder form by a mortar and pestle to obtain DCL ECM.

2.3. Preparation of Rat Liver DCL ECM Solution. Frozen DCL ECM lyophilized for 72 h was ground into a fine powder using liquid nitrogen. Pepsin (10% w/v) and 2 mg/mL DCL ECM powder were mixed in the presence of 0.5 N acetic acid and kept at room temperature with constant stirring for 48 h.28 The mixture was then transferred to tubes and centrifuged for 15 min at 3000 rpm. The pellet was discarded, and the supernatant was centrifuged till it was clear. The supernatant was neutralized using sodium hydroxide to ensure removal of remaining particulate matter. Until further use, the resulting DCL ECM solution was stored at −80 °C (Figure 1d).

2.4. Analysis of ECM Components/Characterization of Decellularization. Characterization of decellularization was performed for histological analysis and nuclear staining. To characterize the DCL, small pieces of tissues were randomly cut from DCL and fixed with 4% formaldehyde, dehydrated, and embedded in paraffin. Tissue sections were deparaffinized and stained with hematoxylin and eosin (H&E). Briefly, DCL sections were first fixed for 4 h in 4% formaldehyde at RT, washed with PBS, dehydrated in graded alcohol series, and subsequently embedded in paraffin. For histological staining, 6 mm thick sections were stained with H&E and examined under a light microscope. For nuclear staining, the paraffin sections were dewaxed in xylene and immersed in 0.1% Triton-X (v/v) diluted in PBS for 2 min. After three consecutive washes in PBS, the specimens were treated with 10% bovine serum ALB for 1 h at 37 °C and subsequently stained with 4′,6-diamidino-2-phenylindole (DAPI) for 2 min at RT for nuclear staining. Finally, the specimens were washed thoroughly in PBS and mounted on glass slides. The images were captured under a fluorescence microscope (Leica DFC295, Germany) using the Leica software application suite (LAS V3.8).

2.5. Preparation of SG and SG–DCL Inks. First, 5% w/v silk–8% w/v gelatin ink was prepared as reported previously by us.20,23 Briefly, sterilized gelatin was dissolved in an autoclaved silk solution held at 37 °C. Then, a solution of Eagle’s minimum essential medium (10%, 10X), 10% fetal bovine serum (FBS), and 10% DCL was also added to the SG mixture, and the ink was homogenized to prepare SG–DCL ink. Further, the same composition without DCL was used to prepare SG ink that acted as control. Mushroom tyrosinase (800 units/mL) was added to both the inks for induction of enzymatic cross-linking. SG–DCL (2 mL) and SG inks were made into films and fixed by 70% ethanol to draw a comparison with the 3D printed scaffolds.

2.6. Characterization of SG–DCL and SG Inks. 2.6.1. Rheological Characterization. DCL (5%) ink and SG–DCL ink with DCL percentages of 1%, 5%, and 10% were analyzed using Anton Paar MCR-302 (Austria). The flow behavior was evaluated in the shear rate range of 0.1–1000 s⁻¹ to analyze the effect of shear on different inks at 22 °C. The storage and loss moduli were attained in the amplitude mode for 0.1–100 rad/s by keeping a constant strain of 1%. Based on the results, modulus behavior of SG and SG–10DCL cross-linked with tyrosinase was also studied. Hereafter, SG–10DCL will be mentioned as SG–DCL.

2.6.2. Fourier Transform Infrared Spectroscopy. Fourier transform infrared (FTIR) spectra (Thermo Electron Scientific, Waltham, MA, USA) of 10% DCL (in deionized water), SG, and SG–DCL cross-linked with the tyrosinase scaffold were captured in absorbance mode, with a spectral resolution of 4 cm⁻¹ and the number of scans of 50, and then, the spectra were analyzed for a relative comparison. All scans were done at the same spot of the same piece of material for each sample. Spectra were plotted using OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA).

2.7. Preparation of SG and SG–DCL 3D Printed Scaffolds. SG and SG–DCL CAD structural parameters for printing are 6 mm × 6 mm and 6 layers, making the total height of the scaffold to be 0.32 mm. The interfilament distance was kept as 1 mm (Figure 2). The SG and SG–DCL (10% SG–DCL) inks were filled in a sterile barrel sealed with a pneumatic piston. The extrusion was carried out at pressure 18–22 psi and at 22 °C to have smooth filamentous extrusion. Extrusion was carried out using a sophisticated direct-write assembly (Fiber Align, Aerotech Inc., Pittsburgh, USA) through a 260 μm microcapillary nozzle at a speed of 1 mm/s.29,30 The 3D printed scaffolds were allowed to cross-link for 30–45 min to allow complete action of the tyrosinase present in the ink. After cross-linking, the 3D printed scaffolds were immersed in PBS and stored at 4 °C until the start of cell culture.

Figure 2. Structural parameters of the 3D scaffold. (a) Top view of the CAD model. (b) Isometric view of the CAD model. (c) Representative image of the 3D printed scaffold. (d) Microscopic image of the 3D printed scaffold.
2.8. Characterization of SG and SG–DCL 3D Printed Scaffolds. 2.8.1. Degradation of SG and SG–DCL 3D Printed Scaffolds. The degradation study of 3D printed SG and SG–DCL scaffolds was carried out in the simulated body fluid (SBF). SBF was prepared as mentioned in our previous study under constant stirring at 37 °C and buffered at pH 7.4 using 75 mM Tris buffer.\(^1\) The samples were kept in the SBF solution at 37 °C in an incubator for 6 days, and the SBF was changed after 48 h. Microscopic images were taken at 24, 72, and 144 h using a Leica DM300 microscope to evaluate the structural stability and the filament diameters to calculate any degradation. Filament diameters were calculated using ImageJ (NIH, USA), and the structures were analyzed for any changes to analyze sample degradation. The loss in diameter of the filament was calculated by the following formula:

\[
\text{loss in diameter} = \left(\frac{\text{initial diameter} - \text{final diameter}}{\text{initial diameter}}\right) \times 100
\]

2.8.2. Swelling of SG and SG–DCL 3D Printed Scaffolds. To check the stability of the 3D printed scaffolds calculated as a measure of changes in the diameter upon swelling, single-layered 3D printed SG and SG–DCL scaffolds were used to measure the changes in the diameter.\(^2\) First, the scaffolds were prepared and kept in air for 40 min and microscopic images were obtained at 10 different points of the same scaffold to calculate the changes in the filament diameter after postprinting using a Leica DM300 microscope. After that, the scaffolds were dipped in PBS and microscopic images were taken at 10 different sites at 22 and 46 h, and filament diameters were measured using ImageJ (NIH, USA). Further, the swelling ratio was calculated using the following formula for percentage swelling:

\[
\% \text{ swelling} = \left(\frac{\text{diameter after swelling} - \text{diameter before swelling}}{\text{diameter before swelling}}\right) \times 100
\]

2.9. Isolation, Characterization, and Expansion of Primary Hepatocytes. Hepatocytes were isolated from male wild-type control mice (C57Bl6/J, 8 week, male, n = 5) by nonrecirculating collagenase perfusion through the portal vein as previously described in our study.\(^3\) Livers were perfused in situ with 45 mL of Gibco Liver Perfusion Media (Invitrogen, Carlsbad, CA) followed by 45 mL of Gibco Liver Digestion Media (Invitrogen). The liver was excised, minced, and strained through a steel mesh sieve. The dispersed hepatocytes were collected by centrifugation at 50 g for 2 min at 4 °C and washed twice with Williams’ media. The final pellet was resuspended in Williams’ media. Hepatocytes were counted, and viability was checked by trypan blue exclusion.

Human liver hepatoma Huh7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) with 10% FBS (HyClone) and 100 μg/mL streptomycin and 100 IU/mL penicillin (pen–streptomycin) (Gibco) at 37 °C in an incubator containing 5% CO\(_2\).

2.9.1. Cell Culture on 2D Films and 3D Printed Scaffolds. Prior to cell seeding, 2D films of SG and SG–DCL and 3D printed SG and SG–DCL scaffolds were incubated in DMEM consisting of 1% pen–streptomycin overnight. The Huh7 cells were then seeded on the 2D films and 3D printed scaffolds at a cell density of 50,000 cells/500 μL and incubated in DMEM consisting of 10% FBS, 1% pen–streptomycin, and 1% ITS (insulin–transferrin–selenium). Microscopic images were taken on days 0, 3, 5, 7, and 14.

After seeding the Huh7 cells on 3D printed scaffolds, the cells were stained with DAPI for nuclear staining and visualized using a fluorescence microscope (Nikon Ti Eclipse).

2.10. MTT Assay. MTT assay was performed to understand the metabolic activity of Huh7 cells on day 3 and day 7. For this, MTT tetrazolium salt was added to the 2D cells or 3D constructs (n = 3) for 4 h in a 1:10 ratio in media followed by dimethyl sulfoxide dissolution. Absorbance values were measured at 570 nm using a microplate absorbance reader (Synergy/H1, BioTek). Scaffolds without cells were treated as a negative control. For statistical comparisons, cells cultured in 2D SG (at day 1) were used as controls.

2.11. Gene Expression Analysis. Total RNA from cells seeded on 2D films and 3D printed scaffolds was isolated by the Trizol-chloroform method and quantified at 260/280 nm with a Thermo Scientific Nanodrop 2000 spectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA with reverse transcriptase (Thermo Scientific Verso cDNA synthesis kit) according to manufacturer’s instructions. Quantitative real-time PCR was performed with SYBR green PCR master mix (Fermentas Life Sciences) on a Viia7 instrument PCR system (Applied Biosystems, USA). The following cycling parameters are used: start at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 60°C for 30 s, elongation at 72 °C for 30 s, and a final 5 min extra extension at the end of the reaction and repeat for 40 amplification cycles. The primer used were for 18 s (housekeeping gene), ASGR1, EpCAM, Lgr5, and β-catenin (Table 1).

### Table 1. Details of the Primers Used for Gene Expression Analysis

<table>
<thead>
<tr>
<th>primer</th>
<th>gene expression analysis</th>
<th>company name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASGR1</td>
<td>asialoglycoprotein receptor 1</td>
<td>Eurofins Scientific</td>
</tr>
<tr>
<td>EpCAM</td>
<td>epithelial cell adhesion molecule</td>
<td>Eurofins Scientific</td>
</tr>
<tr>
<td>Lgr5</td>
<td>leucine-rich repeat-containing G-protein coupled receptor 5</td>
<td>Eurofins Scientific</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>(catenin beta 1/β-catenin)</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>

2.12. Immunofluorescence Analysis. For immunofluorescence (IF) staining, 4 μm thick cryosections from the 3D scaffolding Huh7 cells on the top layer were cut and hydrated with 0.5% BSA–PBS for 15 min. Sections were then exposed to blocking buffer (0.1% donkey serum–PBS) for 30 min. Next, primary ASGR1 antibody (rabbit, Santa Cruz, 1:50) and ALB (mouse, Santa Cruz, 1:50) were incubated overnight at 4 °C. After washing, cryosections were incubated with antirabbit FITC (Santa Cruz), and antimouse FITC (Santa Cruz) secondary antibodies for 45 min. Finally, sections were mounted using with a DAPI mount. The staining images were acquired with a fluorescence microscope (Nikon Ti Eclipse). Quantification of cells was done using NIS-Elements software.

2.13. Liver Function Test. Liver-specific functions of the hepatic cells cultured on the bioprinted scaffolds were studied by measuring ALB and urea secretion in the cell culture supernatants. ALB was measured using a commercially available ELISA kit (Sigma-Aldrich) designed for the quantitative measurement of ALB levels in cell culture conditioned media. Urea in cell culture supernatants was measured calorimetrically at 570 nm using the urea assay kit (Sigma-Aldrich).

2.14. Statistical Analysis. All quantitative data were expressed as mean ± standard deviation. Statistical significance was accepted as p ≤ 0.05(*) and p ≤ 0.001(**). Student’s unpaired t-tests were used to analyze and compare the groups. All experiments were repeated at least three times.

3. RESULTS

3.1. Characterization of DCL ECM. Characterization of DCL was performed to assess the efficiency of decellularization for the presence of remnant cells. The histology-based microscopic analysis revealed a complete absence of cells in histology in the DCL. The vascular structures were however intact and well visible in the histology (Figure 1e). DAPI analysis further validated the absence of cells in the DCL matrix (Figure 1f).
3.2. Rheological Characterization of SG–DCL and SG Inks. Silk (5%) and 8% gelatin with various concentrations of the DCL (1%, 5%, and 10%) were assessed for their rheological behavior (Figure 3). All of the SG–DCL compositions demonstrated shear thinning behavior (Figure 3a) and provided postprinting hydrogel stability. Compared to 1% and 5% DCL-containing bioinks, the highest concentration (SG–10DCL) bioink exhibited pronounced shear thinning. As the bulk polymer concentration in the bioink increased, the onset of shear thinning shifted toward lower values of shear rates. DCL (5%) alone showed liquidlike behavior, as the frequency-dependent loss modulus, \( G''(\omega) \), was higher than the frequency-dependent storage modulus, \( G'(\omega) \), at lower angular frequencies and almost similar at higher angular frequencies (Figure 3b). However, after mixing with silk–gelatin, all bioinks showed gel-like behavior (\( G' < G'' \)). At a small amplitude oscillatory shear, \( G'(\omega) \) and \( G''(\omega) \) values of the SG–DCL bioinks seemed to show a similar slope. However, frequency-dependent storage modulus reached the plateau (Figure 3c–e). Additionally, on increasing the concentration of the DCL in the SG ink, the gap between the loss and storage moduli widened. This gap was maximum.
for the 10% DCL in the SG ink, therefore making it the most suitable composition for the ink (Figure 3e).

After inducing covalent cross-linking by tyrosinase enzyme, the $G'$ and $G''$ increased by 100 times at 100 rad/s in response to the changes in frequency for the SG–DCL and SG bioinks (Figure 3f,g), particularly at higher angular frequencies, due to effective cross-linking between the protein polymer chains. The $G'$ values exceeded the $G''$ values in the whole angular frequency range ($G' > G''$). These results demonstrated the formation of a printable hydrogel bioink structure. At lower angular frequency values, unchanged loss modulus indicated the rubber plateau zone.

3.3. FTIR Analysis of SG–DCL and SG Inks. First, the amide I region was observed in all of the spectra in the range of 1600–1700 cm$^{-1}$ (Figure 4). Similarly, amide II and amide III

![Figure 4. FTIR spectra of the DCL matrix, SG, and SG–DCL.](image)

regions were observed in all three samples. Further, the region of the N–H protein at around 3300 cm$^{-1}$ was dominated by the highest intensity in SG–DCL as compared to other samples. The alkyl group stretch was more prominent in the DCL due to the presence of fats in the samples, whereas it was absent in SG and SG–DCL. The peak at 1080 cm$^{-1}$ was observed in both DCL and SG–DCL, representing the peak for phospholipids.

FTIR spectra of DCL ECM indicated the presence of collagen chains. DCL ECM showed all of the characteristic peaks of amide I (C=O stretching), II (N–H deformation), and III (N–H deformation) bands at 1643, 1523, and 1232 cm$^{-1}$, respectively. Moreover, the amide B region is visible at 3286 cm$^{-1}$, which indicated stretching vibrations of N–H in phospholipids, glycolipids, and fatty acids. The peaks at 1421, 1340, and 1086 cm$^{-1}$ are representative of the presence of the DNA or RNA content in it. These peaks are representative of the phosphate group present in nucleic acids. Moreover, the peaks of Triton-X (3412, 2951, 2871, 1610, 1512, and 1120 cm$^{-1}$) and SDS (1075 cm$^{-1}$) used for liver decellularization were absent. This indicates the complete removal of the cytotoxic components from the DCL ECM during the postdecellularization process.

Peaks at 526, 1084, and 1232 cm$^{-1}$ are present in DCL and SG–DCL absent in SG spectra. The peak at 526 cm$^{-1}$ indicates the weak presence of disulfide bonds, which is further validated by a confirmative peak at 1075 cm$^{-1}$. However, in both our plots, there is no peak in this region, confirming disulfide bonds, whereas the peak at 1084 cm$^{-1}$ is for the strong C–O stretching present on the primary alcohol, which is elevated in the SG–DCL ink. As the major component of DCL is stated to be collagen, this peak is indicative of primary alcohols present in serine, threonine, tyrosine, and phenylalanine. The peak at 1232 cm$^{-1}$ is for the C=O stretching, which indicates the presence of the protein in the particular sample. The peak intensity is recorded maximum for the SG–DCL composition. The peaks at 2925 and 3080 cm$^{-1}$ are present only in the DCL. The peak at 2925 cm$^{-1}$ indicates the presence of the C–H stretching of alkane groups, whereas the peak at 3080 cm$^{-1}$ is for the C–H stretching of alkene groups. The broad peak observed at 3282 cm$^{-1}$ for all of the plots is for the C–H stretching of alkynes. These three peaks represent the bonding present in DCL, SG, and SG–DCL.

3.4. Characterization of SG and SG–DCL 3D Printed Scaffolds. 3.4.1. Degradation. With an increase in the time interval, the filament diameter decreased for both SG and SG–DCL 3D printed scaffolds (Figure 5A). The calculated values showed that there was a 6.09 ± 0.84% loss in the filament diameter after 144 h for SG–DCL scaffolds and a 5.42 ± 1.0% loss in the diameter after 144 h for SG scaffolds (Figure 5B). Enhanced degradation in the case of SG–DCL scaffolds may be due to the presence of DCL ECM in the scaffolds.

3.4.2. Swelling Ratio. We observed a 3.81 ± 0.494% decrease in filament diameter as the scaffolds were kept for
drying at room temperature. Further, there was 5.19384 ± 1.83% swelling observed after 22 h of PBS immersion (Figure S1).

3.5. In Vitro Cell Culture. To determine cell attachment and proliferation on 3D printed scaffolds, we seeded liver hepatoma Huh7 cells on them. Cell attachment was seen on the 3D printed scaffolds within 2 h of cell seeding, which was indicative of good affinity. In terms of cell attachment, we did not observe much difference between the 3D SG and SG−DCL scaffolds on day 0. Cell attachment, spread, and growth on the 3D printed scaffolds were much better in comparison to those observed in the 2D films (Figure 6Aa). The cell growth and density were however increased around days 3 and 7 on the SG−DCL scaffolds as compared to those on the 3D SG scaffolds (Figure 6Ab). DAPI staining on 3D printed scaffolds clearly showed an increase in the number of cells seeded on SG−DCL scaffolds as compared to SG alone on both day 3 (p = 0.003) and day 7 (p = 0.04) (Figure 6B,C).

3.6. MTT Assay. Results showed that as compared to 2D SG on day 1, there was a significant increase in cellular metabolic activity in all conditions including 2D SG and 2D SG−DCL and also 3D SG and 3D SG−DCL on days 3 and 7 (p < 0.05 for each) (Figure 7A). There was a marked increase in cell proliferation in 2D SG−DCL as compared to that in 2D SG on both days 3 and 7 (p < 0.05); however, we obtained
similar results with 3D SG and 3D SG–DCL in terms of metabolic activity on both these days.

3.7. Gene Expression Analysis of mRNA. The gene expression analysis (Figure 7B) was performed to assess the expression of asialoglycoprotein receptor 1 (ASGR1), a mature hepatocyte marker, and EpCAM, a hepatic stem cell marker. For ASGR1, there was no significant change in its expression for both 2D SG and 2D SG–DCL on day 3 as well as day 7 as compared to day 0 2D monolayer control hepatoma cells, while there was 4 ± 0.02-fold (p < 0.05) upregulation in 3D SG and 6 ± 0.04-fold (p < 0.05) upregulation in 3D SG–DCL in its expression. Further, on day 7, there was 9 ± 0.1-fold (p < 0.05) upregulation in 3D SG and 18 ± 0.5-fold (p < 0.001) upregulation in 3D SG–DCL. On day 14 too, there was a significant increase in the expression of ASGR1 in both 3D SG (10 ± 1.2, p < 0.05) and 3D SG–DCL (20 ± 1.1, p < 0.001) in comparison to the controls, similar to day 7 cells in these conditions. Overall, 3D printed constructs revealed upregulated expression of ASGR1 as compared to 2D films, with 3D SG–DCL showing the maximum upregulation of all of the conditions. Interestingly, the expression of ASGR1 in Huh7 cells cultured on 3D SG–DCL was comparable to that of the primary rat hepatocytes cultured on collagen-coated plates on day 3 (Figure S2).

In the case of EpCAM, there was 13 ± 0.3-fold (p < 0.001) upregulation in 2D SG and 6.8 ± 0.2-fold (p < 0.05) upregulation in SG–DCL on day 3 as compared to day 0 2D monolayer control cells, while the expression was upregulated by 19 ± 0.6-fold (p < 0.001) in 3D SG and 6.1 ± 0.1-fold (p < 0.05) in 3D SG–DCL as compared to day 0 2D monolayer cells. Further, on day 7, there was a decrease in the expression of EpCAM for all of the conditions, except for 3D SG, which showed an upregulation of 4.5 ± 0.04-fold in comparison to the control cells. Overall, 2D SG–DCL and 3D SG–DCL showed the lowest expression and 3D SG demonstrated the maximum expression on day 7. The expression of EpCAM was further reduced by about 5 ± 0.2-fold on day 14 in cells plated on 3D SG–DCL as compared to the controls. EpCAM expression was however significantly less in the primary rat hepatocytes as compared to that in the Huh7 cells.

The canonical Wnt pathway-specific marker β-catenin was upregulated by more than 4 ± 0.7-fold in 3D SG–DCL on day 3 (p < 0.05), while it showed a 50% decline on day 7 as compared to 2D controls (p < 0.05). The gene expression of β-catenin was the highest in cells cultured on 3D SG on day 7. The Wnt-responsive gene Lgr5 also exhibited a similar trend with increased expression on day 3 and a reduced expression on day 7 in the 3D SG–DCL scaffolds in comparison to the 2D control. On day 7, however, the decrease in Lgr5 expression was not significant as compared to the controls. The expression of β-catenin and Lgr5 genes in the 3D SG–DCL scaffolds, although not significantly different than the controls, was reduced on day 14 in comparison to that observed on day 3, similar to the trend seen on day 7.

3.8. IF Analysis. To validate the protein expression of hepatocyte-specific proteins, IF studies were performed on day 7 for both 3D SG and SG–DCL cell-seeded scaffolds (Figure 8). The protein expression of the cell-specific phenotype
marker ASGR1 was increased in cells plated on 3D SG–DCL on day 7 in comparison to that observed in cells plated on 3D SG scaffolds. The expression of another functional marker ALB was not observed in the 3D SG scaffolds, while its expression was well evident in cells grown on 3D SG–DCL scaffolds.

3.9. Liver Function Test. The liver function test was performed to assess the levels of ALB and urea produced by the cells cultured on 2D SG, 2D SG–DCL, 3D SG, and 3D SG–DCL on days 3 and 7 (Figure 9). In comparison to 2D SG, ALB production was enhanced in cells cultured on SG–DCL in both 2D (2-fold, $p < 0.05$) and 3D (2.5-fold, $p < 0.05$) conditions on day 3 of culture. On day 7, ALB production was increased in all conditions in comparison to 2D SG with maximum production in 3D SG–DCL (3-fold, $p < 0.05$). Urea production was also maximally enhanced in cells cultured on 3D SG–DCL on day 3 (10-fold, $p < 0.05$) and day 7 (6-fold, $p < 0.05$) as compared to cells on 2D SG scaffolds. Overall, liver functions were maximally observed in the combinatorial ink of DCL and SG in both 2D and 3D conditions. Also, urea secretion was similar in these two conditions. ALB levels were

Figure 8. (a) Immunostaining of ASGR1 and ALB on 3D scaffolds of SG and SG–DCL on day 7. Dot plots showing the number of fluorescence signal Huh7-positive cells plated on SG and SG–DCL scaffolds on day 7 of (b) ASGR1 and (c) ALB. Quantification of cells was performed using NIS-Elements software.

Figure 9. Liver function test for ALB and urea secretion for 2D films and 3D printed scaffolds of both SG and SG–DCL on days 3 and 7 (** for $p < 0.05$, *** for $p < 0.001$; $p$ values have been calculated by comparing all groups with 2D SG on days 3 and 7).
however higher in primary rat hepatocytes in comparison to those secreted by Huh7 cells cultured on 3D SG−DCL.

4. DISCUSSION

Several studies have used the DCL ECM in a solubilized form to generate 3D injectable hydrogels.\textsuperscript{7,11} The viability and functionality of hepatocytes have been shown to be significantly improved in DCL hydrogels compared to those observed in collagen hydrogels.\textsuperscript{39} Recent studies have also reported 3D bioprinting of liver cells, in which bioprinted hepatocytes and nonparenchymal cells in 3D architecture have been reported to be metabolically and functionally active.\textsuperscript{40,41}

In another study, the researchers have used a custom-made inkjet 3D bioprinter for printing two layers of hepatocytes using the galactose chain of galactosylated alginate gel as the bioink stimulated hepatocyte adhesion and assisted in maintenance of cellular polarity.\textsuperscript{42} Yang et al. reported the construction of 3D bioprinted hepatorganoids using HepaRG cells and gelatin bioinks and showed their therapeutic potential in the mice model of liver failure.\textsuperscript{43} The major challenge of using DCL for 3D printing is that it does not exhibit optimum biophysical properties required for a printable fluid. A few attempts have been made to use polycaprolactone or other degradable thermoplastic polymers as temporary support material during printing, but this approach is problematic due to the need for extensive chemical treatment to remove that component in a later stage. For using DCL in 3D printing techniques, the physical, biological, and mechanical properties of DCL need to be optimally modified. Second, liver tissue-derived ECM-based scaffolds may provide tissue-specific signals for liver regeneration. Our previous findings indicated that the SF protein can upregulate the Wnt/β-catenin signaling pathway,\textsuperscript{22,23} which is crucially important for liver regeneration. A pertinent question is how to develop a printable biomimetic ink by combining liver-derived ECM and SF protein.

A thorough rheological characterization is required for the development of a bioink. The primary criterion for the bioink is extrusion and shape retention capability.\textsuperscript{44} As observed, 5% DCL solution lacked printability and the sol−gel transition property. All of the SG−DCL compositions demonstrated shear thinning behavior and provided postprinting hydrogel stability. Stability of the SG−DCL scaffold, i.e., storage and loss moduli, was studied. The storage modulus was higher for all of the SG−DCL compositions, but the difference in storage and loss modulus values increased with an increase in DCL concentration in SG−DCL. Therefore, we chose SG−10% DCL bioink for developing 3D printed scaffolds, since the storage modulus was higher than the viscous modulus at all of the given points. Simultaneously, SG−10DCL has the lowest loss modulus values. Our findings are comparable to the findings of Zhang et al., who used cartilage-derived DCL. They reported that 2−3% DCL and 2.5−7.5% SF protein were most appropriate for cell-laden bioprinting and the G’ values exceeded the G” values over the whole angular frequency range characterized.\textsuperscript{44,45}

Tissue decellularization can be performed by ionic or nonionic detergents in the presence of different physical forces, such as perfusion, orbital shaking, or performing repetitive freeze−thawing of the tissue sample.\textsuperscript{46} These methods are found to affect the conformational structure of the native fibrous ECM as they disrupt or solubilize the cellular components over the period of reaction, i.e., about 24 h.\textsuperscript{46−49} Therefore, in the current study, a gradient of Triton-X and SDS was perfused directly through the hepatic vascular system of the rat liver. A similar approach reported earlier led to the minimal disruption of the secondary conformation of the tissue structure; moreover, it decreased the time of decellularization to about 90 min.\textsuperscript{50} Perfusion through the hepatic vascular system has been also performed for the whole human liver samples using a composition of Triton-X and ammonium hydroxide. Complete decellularization took place in 4−6 days, but the ECM remained intact.\textsuperscript{51} In our study, FTIR spectra indicated minimal damage to ECM, as there is only single peak in the amide I region (1600−1700 cm\textsuperscript{−1}) at 1643 cm\textsuperscript{−1}, indicative of the α helical structure, as well as preservation of the protein content of the liver (Figure 4).

We fabricated a novel bioink by a combination of solubilized native DCL and SF protein to obtain an ideal liver-specific scaffold. There was a marked increase in cell proliferation in 2D SG−DCL as compared to 2D SG on both days 3 and 7 (p < 0.05); however, we obtained similar results with 3D SG and 3D SG−DCL in terms of cell viability on both these days. We showed that the novel bioink led to a significant enhancement of liver-specific marker ASGR1 and functions including ALB and urea secretion. ASGR1 is one of the recognition motifs on the surface of hepatocytes, which promotes their adhesion to ECM in liver tissues and appropriate artificial surfaces. The increase in the expression of ASGR1 on SG−DCL scaffolds revealed significant improvement in cell−cell communication and proliferation, thereby demonstrating the positive influence of the novel biomaterial on ASGPR-mediated adhesion of hepatic cells.\textsuperscript{52} The presence of DCL ECM in SG−DCL scaffolds stimulated prolific tissue remodeling via mechanical strength, ECM composition, and cellular proliferation and differentiation.\textsuperscript{53}

The silk-based bioink has been reported earlier to show a drastic upregulation of Wnt/β-catenin signaling pathways.\textsuperscript{20,27,54} Wnt/β-catenin tightly controls the molecular pathway that regulates cell fate during embryogenesis and hepatobiliary development, as well as liver homeostasis and repair in adulthood. Abnormal Wnt/β-catenin signaling, as well as mutations in key regulatory genes of the Wnt/β-catenin pathway, promotes the development and progression of different liver diseases, including cancer and hepatobiliary tumors.\textsuperscript{25,26} In our study, we observed a substantial upregulation in the expression of the Wnt pathway gene β-catenin and Wnt-responsive gene Lgr5 in Huh7 cells cultured on both SG and SG−DCL in 3D formats on day 3 of cultures, while a decline of these genes on days 7 and 14 was also observed. It has been earlier documented that Huh7 cells significantly express Lgr5 that induces epithelial−mesenchymal transition and hence tumor metastasis via the Wnt/β-catenin pathway.\textsuperscript{55} Wnt/β-catenin is also known to play a significant role in expansion of hepatoblasts or hepatic progenitors during early liver development.\textsuperscript{25,26,56} An upregulation in the expression of both EpCAM and β-catenin on day 3 and then a significant decline in 3D SG−DCL are indicative of the fact that the liver-specific matrix might be modulating the Wnt pathway possibly via mechanical signals.\textsuperscript{57}

In contrast to ASGR1, the expression of EpCAM was increased in SG and SG−DCL scaffolds on day 3 in comparison to controls and its expression declined on day 7 in these conditions. EpCAM marks human liver progenitor cells, which display characteristics of liver stem/progenitors, and is absent on mature hepatocytes. Interesting expression of
Lgr5, which also is a characteristic marker of hepatic progenitor cells, was downregulated in SG–DCL scaffolds on day 7. Interestingly, the EpCAM expression was the lowest, while that of ASGR1 was the highest on SG–DCL in both 2D and 3D conditions on days 7 and 14, implying that the SG–DCL facilitates the differentiation of mature hepatocytes from the bipotent progenitors. It should be noted that the primary hepatocytes also showed a significantly decreased expression of EpCAM in comparison to that observed in Huh7 cells plated on 3D SG–DCL on day 3.

Although Huh7 cells demonstrated high proliferation in both 2D and 3D conditions, the hepatocyte gene expression and specific functions (ALB and urea secretion) were increased more in 3D printed constructs compared to those in 2D culture conditions. This increase in hepatic gene expression and functions on 3D printed constructs indicates the relevance of 3D geometry on cellular functions. This disparity between gene expression and protein function in simple 2D culture models suggests that our 3D printed scaffolds, especially the 3D SG–DCL, mimicked the physiological environment of the native liver most closely and thus promoted both hepatic expression and function in the cultured cells. This strategy offers great advantage over conventional in vitro hepatocyte culture in sandwich culture conditions, in which cells are cultured between two layers of collagen type I and Matrigel. Sellaro et al. reported that hepatocytes cultured in sandwich culture in porcine-liver-derived DCL ECM produced a similar level of ALB secretion as well as ammonia metabolism to those cultured in Matrigel. Our findings demonstrated that 3D printed SG–DCL constructs in vitro could offer further elevation of hepatocyte-specific functionalities and that these constructs could be maintained for longer culture duration.

Our method amalgamates DCL, gelatin, and SF protein in optimized ratios to create and print the most apt scaffold/environment for the growth of liver cells. Liver regeneration strategies are moving toward development of biomimetic 3D printed vascularized liver constructs using liver cells and natural biomaterials such as gelatin and fibronectin by 3D bioprinting. These studies have displayed critical liver functions, including ALB production, cholesterol biosynthesis, and inducible cytochrome (CYP) 1A2 and CYP 3A4 activities. Lee et al. also developed a DCL ECM bioink for 3D cell printing of HepG2 cells and illustrated enhanced hepatic differentiation and functions of the cells. The next step would be to 3D bioprint the primary liver cells and other support cells along with the prepared SG–DCL bioink together to replicate the in vivo liver tissue environment. It will be interesting to understand how modulation in viscoelastic behavior of the SG–DCL bioink can enhance liver-specific ECM synthesis by the liver cells.

5. CONCLUSIONS
In summary, we demonstrate the printability of a novel hybrid SG–DCL bioink. SG–DCL 3D printed scaffolds provided a conducive microenvironment for maintaining differentiation and functions of hepatic cells through the activation of the Wnt/β-catenin signaling pathway. The 3D printed constructs developed using the SG–DCL bioink elevated regulation of liver tissue-specific gene and protein expression during in vitro culture of hepatocytes compared to 2D culture models or only SG constructs. The developed liver-specific bioink has immense potential for 3D printing applications in liver tissue engineering.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.1c00671.

AUTHOR INFORMATION
Corresponding Authors
Savneet Kaur — Institute of Liver and Biliary Sciences, New Delhi 110070, India; Email: savykaur@gmail.com
Sourabh Ghosh — Regenerative Engineering Laboratory, Department of Textile and Fiber Technology, Indian Institute of Technology-Delhi, New Delhi 110016, India; orcid.org/0000-0002-1091-9614; Email: sourabh.ghosh@textile.iitd.ac.in

Authors
Aarushi Sharma — Regenerative Engineering Laboratory, Department of Textile and Fiber Technology, Indian Institute of Technology-Delhi, New Delhi 110016, India
Preety Rawal — Institute of Liver and Biliary Sciences, New Delhi 110070, India
Dinesh M Tripathi — Institute of Liver and Biliary Sciences, New Delhi 110070, India
Dashrath Alodiya — Regenerative Engineering Laboratory, Department of Textile and Fiber Technology, Indian Institute of Technology-Delhi, New Delhi 110016, India
Shiv K Sarin — Institute of Liver and Biliary Sciences, New Delhi 110070, India

Author Contributions
A.S. and P.R. contributed equally to this work.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This work was supported by intramural funding from IIT Delhi and ILBS.

REFERENCES
(4) Vinken, M.; Maes, M.; Oliveira, A. G.; Cogliati, B.; Marques, P. E.; Menezes, G. B.; Dagli, M. L. Z.; Vanhaecke, T.; Rogiers, V.


