



Effect of varying cell densities on the rheological properties of the bioink

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ABSTRACT

The rheological characterization of any biopolymer solution is crucial for evaluating the overall printability or injectability of the hydrogel. However, the effect of cells in the cell-laden hydrogel's rheological profile is often ignored. As a result, there is a significant difference in the predicted and experimental outcome in the structural stability of the construct as well as on the cell viability, proliferation, and differentiation potential of the embedded cells. Our present study has addressed the effect of different cell densities (0.1 million cells/ml, 0.5 million cells/ml, 1 million cells/ml and 2 million cells/ml) of TVA-BMSCs on the flow property, modulus behaviour, gelation kinetics and printability of our proprietary silk fibroin-gelatin (5SF-6G) bioink. The cell-laden hydrogels demonstrated a characteristic shear thinning behaviour (low initial viscosity), low storage modulus and increased gelation time when compared to the acellular 5SF-6G hydrogel. The printability analysis also portrayed a square pore geometry with low spreading ratio in 1 million cells/ml encapsulated 5SF-6G hydrogel comparable to the acellular hydrogel. We postulated that incorporation of cells in the bioink interfered with the gelation mechanism of the mushroom tyrosinase in the 5SF-6G bioink by masking the active sites. Additionally, the mechanistic crosstalk between the cell-surface integrins with the cell-attachment motifs of the biomaterial alters the cellular biomechanics of the cell that in-turn profoundly impacts the rheological properties of the polymer blend. Therefore, cell density of 1 million cells/ml was considered the best fit for extrusion-based 3D bioprinting owing to its optimum rheological traits and printability index akin the acellular hydrogel.

1. Introduction

3D bioprinting is an advanced biofabrication technology for designing complex tissue architecture through precise patterning of cells, growth factors or signalling molecules and polymeric materials in a layer-by-layer fashion onto a solid substrate. The process involves a wide range of complexities from the choice of biomaterial, design, cell type selection, biomaterial characterization (physical and rheological) and preference of 3D bioprinting technique to achieve optimum printability and cyto-compatibility [1,2]. Extrusion-based 3D bioprinting is the most versatile biofabrication tool used by tissue engineers worldwide due to its broad range of material viscosity, the varying molecular weight distribution of biomaterials enabling fabrication of layered constructs with high cell density and cell viability [3,4]. The challenging part lies in designing the bioink that provides valuable insights into the rheological and mechanical characteristics of the 3D bioprinted constructs ensuring high-resolution cellular deposition [5,6].

The rheology profile of any hydrogel plays a determining role in the flow properties [7], deformation characteristics [8], shape fidelity [9]

and thus the overall injectability or printability of the bioink [5,6]. Additionally, the rate of cell proliferation and differentiation is also dependent on the rheological behaviour of the bioink [10]. Therefore, for any extrusion-based additive manufacturing process, rheological characterization of the proposed biomaterial in terms of viscosity, yield stress, time-dependent and independent viscoelastic properties, and gelation kinetics is directly interlinked with the mechanical stability and bioactivity of the polymer post extrusion [11,12]. Mouser et al. have elucidated the role of yield stress as the primary regulatory factor in any 3D bioprinting approach by using different concentrations of gelatin methacryloyl and gellan gum [13]. Chen et al. also explained the necessity of decreasing viscosity of the bioink with increasing shear rate (shear-thinning behaviour) using a combination of alginate, gellan gum and bioactive molecules [14]. Thus, identifying appropriate yield stress and viscosity is a prerequisite for homogenous mixing of cells in the bioink [15], smooth extrusion of the cell-laden bioink through the nozzle [16] and fabrication of stable self-supporting structures. The viscoelastic properties of the bioink also have an essential role in shielding the cells against shear stress and the overall structural and

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physiological stability of the construct [17]. Gao et al. have extensively investigated the dynamic modulus profiles of the gelatin-alginate blend to conclude the extreme dependency of printing outcome on the viscoelastic properties of the composite biomaterial [18]. Above all, Ouyang et al. used different combinations of gelatin and alginate to define an optimum gelation time of the bioink as a significant parameter in attaining high printability and stable interpenetrating network formation of the hydrogel [19]. These rheological parameters have been widely exploited by researchers worldwide to demonstrate the correlation of rheology with printability using different experimental and computational modelling approaches. Paxton et al. have used different materials like Poloxamer, Alginate, Gelatin and Nivea crème to determine a printability window using a combination of mathematical modelling and different rheological and printing parameters like viscosity, shear rate, nozzle diameter, printing pressure, extrusion velocity [20]. Such mathematical modelling strategies can be utilized to evaluate the extrusion velocity, printing pressure and nozzle speed completely based on the rheological profile of the hydrogel to ensure high cell survival during and post 3D bioprinting [20].

Through the years, with continuous rheological optimization and printability assessment, our laboratory has developed a standardized silk-gelatin bioink using mushroom tyrosinase as the enzymatic crosslinker. This bioink have been mixed with a wide array of cell types like human fibroblasts [21], human bone-marrow derived mesenchymal stem cells [22] or human articular chondrocytes in dispersed or aggregate [23] form to either recapitulate the anatomical microarchitecture of a particular tissue [24] or mimic the embryonic development pathway of a tissue [25] or develop an *in vitro* disease model. Especially Das et al. in 2013 studied the rheological properties of silk fibroin-gelatin blend at different concentrations of silk and gelatin component formed by physical mixing without the use of any crosslinker [26]. However, reports published by our group in 2016 demonstrated the requirement of additional enzymatic crosslinking to enhance the mechanical properties of the bioink. Using mushroom tyrosinase as a crosslinking agent, the viscosity drop and the dynamic modulus characteristics increased by multifold times, depicting higher resolution printability and cell viability [27]. A similar approach has been adopted by several other researchers worldwide using a combination of a wide array of natural and synthetic biomaterials [8,18,20]. A printability analysis was also carried out by Trucco et al. using silk fibroin-gelatin bioink utilizing different printing conditions like nozzle diameter, nozzle speed, needle length and printing pressure [28]. A mathematical model was established based on the flow behaviour of the silk fibroin-gelatin and alginate-gelatin bioinks to calculate the printing pressure, velocity and speed required for smooth extrusion of the filament with optimum diameter and porosity required for nutrient exchange [28]. Although these rheological and printability analyses provide detailed information about the viscosity and the modulus property of the hydrogel required for 3D printing, the effect of the cellular component and its interaction with the biomaterial component on the rheology profile is often ignored due to limitations of cost and time required in cell expansion [5]. This can be a plausible reason for such variation in the rheological characteristics of the final printed construct from the predicted model [29].

Eukaryotic cells are composed of a spanning cytoskeletal network that allow the cells to change shape, migrate or interact with each other. The mechanical properties of these cells arise from a complex network of filamentous proteins (intermediate filaments, actin-myosin network and microtubules), that contributes to an elastic modulus of 1.7 GPa equivalent to keratin [30]. When cells are encapsulated in a 3D hydrogel, the initial phase of cell-hydrogel interaction are mediated by cell-surface expressed proteins like integrins, syndecans, and other cell adhesion molecules [31]. Among them, integrin plays a major role as it binds to the cell-adhesion motifs of the respective hydrogel and regulate the downstream mechanotransduction pathway via actin polymerization through a large set of linker proteins like talin, vinculin, etc

affecting the overall mechanical property of the hydrogel [32]. Moreover, in a cell-laden hydrogel, cells with an approximate diameter of 1 μm is embedded in a hydrogel having a mesh size of 10–100 nm. Thus, cells need to degrade the surrounding hydrogel matrix in order to migrate and homogeneously distribute themselves throughout the 3D niche thereby affecting the mechanical stability of the supporting hydrogel network [33]. In recent years, a handful of rheological studies of cellular bioinks have been carried out. Varying the cell densities substantially affects the flow, viscoelastic, and gelation behaviour of the bioink [34,35]. Interaction of cells with biomaterial has also demonstrated a change in printability of the bioink [36,37]. These cell-matrix interactions are assumed to cause a decline in the viscosity and storage modulus of the bioink [29,34]. However, Xu et al. reported increased viscosity and storage modulus with increasing cell concentrations [38]. Researchers have adopted several mathematical modelling approaches to predict the viscoelastic properties of the cell-laden bioink during cell encapsulation [29,39,40]. This suggests that the interaction of the biomaterial and cell suspension can drastically affect the overall rheological properties of the bioink through various integrin-actin mediated mechanotransduction events altering the mechanical property of the cell and its surrounding matrix [33]. Thus, a concrete conclusion cannot be drawn from the above-published reports on whether increasing cell density in the bioink causes a rise or fall in viscosity and storage modulus of the biopolymer solution. Moreover, the effect of varying cell densities in the enzymatically crosslinked silk fibroin-gelatin bioinks has not been reported.

In our present study, we have analysed the effect of various cell densities (0.1, 0.5, 1 and 2 million cells/ml) of TVA-BMSCs on flow behaviour, viscoelastic properties, and gelation kinetics of enzymatically crosslinked and non-crosslinked silk-gelatin bioink. This immortalized mouse BMSC cell line was engineered to express TVA receptors (SV40 T Antigen) to allow incorporation of genes via avian retroviruses [24]. Additionally, the effect of enzymatic crosslinking on the viscoelastic and flow properties of the cell-laden bioink has also been evaluated. Further, we performed the preliminary printability analysis to determine the suitable cell density for enzymatically crosslinked silk-gelatin bioprinting.

2. Material and methods

2.1. Preparation of silk fibroin solution

5% silk solution was prepared by combining two processes, namely degumming and dissolution, as previously described [41–43]. Briefly, *Bombyx mori*. Silk cocoons obtained from Central Silk Board (Bangalore) were chopped into small pieces using a scissor and boiled two times in 0.02 M Sodium Carbonate (Na_2CO_3 , Fisher Scientific) solution (Degumming). The silk fibers were cleansed 5–6 times in deionized water to remove any residual sodium carbonate or sericin and left to dry overnight at 37 °C. The dry fibroin fibers were dispersed in a 9.3 M Lithium Bromide (LiBr, SRL) solution at 60 °C for 4 h. The LiBr was removed from the fibroin solution by dialyzing against deionized water (Dissolution) and then the pure fibroin solution was stored at 4 °C until further use. Then, the percentage of silk solution obtained was determined by following the similar protocol as elucidated previously [21, 44].

2.2. Acellular silk fibroin-gelatin solution formulation

The acellular bioink was prepared by adding 6% (w/v) ethanol sterilized gelatin (Sigma Aldrich) into autoclaved 5% fibroin solution. Additionally, a mixture of 10 $\times$ media (Himedia) and 10% Foetal Bovine Serum (FBS, Gibco) was added to the already mixed silk fibroin-gelatin solution and incubated at 37 °C until the formation of a clear solution (5SF-6G). To initiate enzymatic cross-linking, 800 U mushroom tyrosinase (Sigma Aldrich) was added to the 5SF-6G blend before the

bioprinting procedure.

2.3. Cellular bioink formulation

TVA-BMSCs were procured from IIT Kanpur. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Himedia), consisting of 10% FBS (Himedia), and 1% Penicillin Streptomycin (Gibco). Upon reaching 80% confluency, the cells were trypsinized, quantified as per our requirement and encapsulated in the already prepared bioink (Section 2.1). Four different cellular concentrations (0.1 million cells/ml, 0.5 million cells/ml, 1 million cells/ml and 2 million cells/ml) were homogeneously mixed with a pipette to prevent cell sedimentation during further experimentation. The cell-laden bioinks were further cross-linked by the addition of 800 U mushroom tyrosinase.

2.4. ATR-FTIR confirmation of mushroom tyrosinase crosslinking

FTIR spectra of crosslinked and non-crosslinked 5SF-6G bioink were obtained in Attenuated Total Reflectance (ATR) mode using a NICOLET - IS-50 FTIR Spectrometer (Thermo Fisher Scientific). The respective spectra were plotted using OriginPro 2021, (OriginLab Corporation, Northampton, MA, USA).

2.5. Rheological analysis

The rheological characterization of different cell-laden and acellular bioinks were carried out using Anton Par MCR 302 plate and plate Rheometer at 25 °C with a plate diameter of 25 mm and spacing of 500 μm . To measure the flow behaviour of the respective bioinks under the influence of shear, the viscosity was evaluated against increasing the shear rate from 0.1 to 10^3 s^{-1} under an environmental cuff. An oscillatory amplitude sweep was performed at a fixed frequency of 5 Hz from 0.1 to 10^4 strain % to evaluate the linear viscoelastic region (LVR) and the respective flow points of different bioinks as a function of storage modulus (G'), loss modulus (G'') and loss factor. The viscoelastic properties of the biopolymer solutions were analysed through an oscillatory frequency sweep test in the LVR from 0.1 to 100 Hz at a constant strain of 1%. The gelation kinetics was investigated by measuring the gelling time/crossover point (T_g) through an oscillatory time sweep experiment at 1% shear strain and 1 Hz frequency.

2.6. Printability assessment

For evaluating the printability, respective cross-linked acellular and cell-laden bioinks were loaded into a sterile 3 ml syringe barrel and inserted in an extruder assembly already fitted to a pneumatic extrusion pump. Here, the printability assessment of cell-laden bioinks was carried out for two cell densities (1 million cells/ml and 2 million cells/ml) as low cell densities (0.1 million cells/ml and 0.5 million cells/ml) were not suitable for developing 3D bioprinted constructs. The bioinks were extruded through a 260 μm microcapillary nozzle using a Direct Write Assembly (Fiber Align, Aerotech Inc., USA). The printing conditions were slightly varied for the respective cellular bioinks owing to their difference in rheological behaviours which are described in Table 1. The printability was calculated by measuring the spreading ratio and

Table 1

Printing parameters for enzymatically crosslinked acellular and cellular 5SF-6G bioinks.

Sample ID	Printing pressure	Printing temperature	Nozzle speed	Number of layers
Acellular	30 PSI	25 °C	1 mm/s	1
1 million cells/ml	22 PSI	25 °C	1 mm/s	1
2 million cells/ml	15 PSI	25 °C	1 mm/s	1

printability index.

2.7. Cell viability analysis using flow cytometry

TVA-BMSCs at a density of 1 million cells/ml were encapsulated in the 5SF-6G bioink prior to 3D bioprinting. Two test sample groups were used for the analysis: a) cells in 5SF-6G bioink before 3D bioprinting and b) cells embedded in 3D bioprinted 5SF-6G construct post-printing. The encapsulated cells were removed from the respective 5SF-6G bioink through protease XIV digestion for 20 min at 37 °C followed by centrifugation at 1500 rpm for 5 min. The cells were then washed with cold PBS and incubated with 50 $\mu\text{g}/\text{ml}$ Propidium Iodide (PI) solution for 30 min in dark at 4 °C. The PI-stained cells were then washed and resuspended in 500 μL cold PBS for further flow cytometry analysis (BD C6 accuri).

2.8. Statistical analysis

All the quantitative data are mentioned as mean $\pm$ standard deviation. One way analysis of variance (ANOVA) was carried out using GraphPad Prism 6 software and $p < 0.001$ (***) is considered statistically significant. All experiments were repeated thrice and in triplicates ($n = 3$).

3. Results

3.1. ATR-FTIR analysis of crosslinked bioink

ATR-FTIR analysis was performed to evaluate the mushroom tyrosinase mediated crosslinking of 5SF-6G bioink (Fig. 1). Strong peaks around 1045 cm^{-1} , 1118 cm^{-1} and 3290 cm^{-1} in mushroom tyrosinase crosslinked 5SF-6G group (5SF-6G-MT) corresponded to the presence of alcohol C-O, C-O-C and phenolic OH groups respectively. Moreover, intensity of these afore-mentioned peaks increased with the oxidation reaction of mushroom tyrosinase enzyme with the 5SF-6G blend [45].

3.2. Effect of cells on the flow behaviour of silk fibroin-gelatin bioink

A comparative analysis between cross-linked and crosslinker-free acellular and cell-laden 5SF-6G bioinks was carried out to elucidate the role of incorporated cells in influencing the flow properties of the blends under the application of increased shear rate. Fig. 2a represents the viscosity vs. shear rate behaviour of crosslinker free silk-gelatin

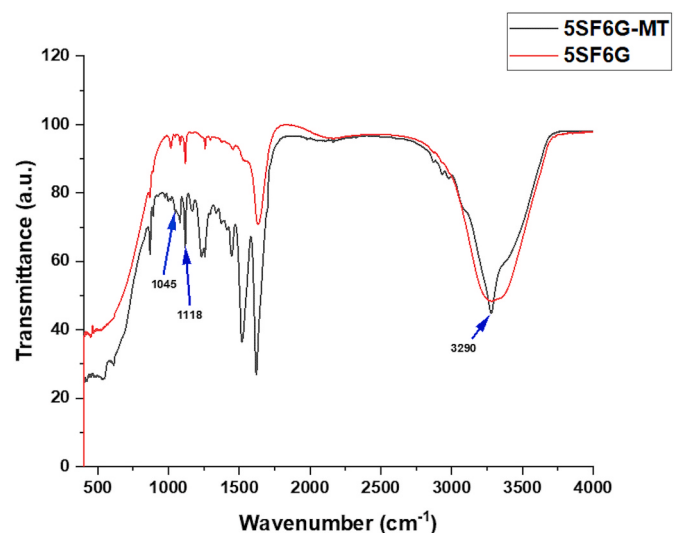


Fig. 1. ATR-FTIR analysis of mushroom tyrosinase crosslinked and non-crosslinked 5SF-6G bioink.

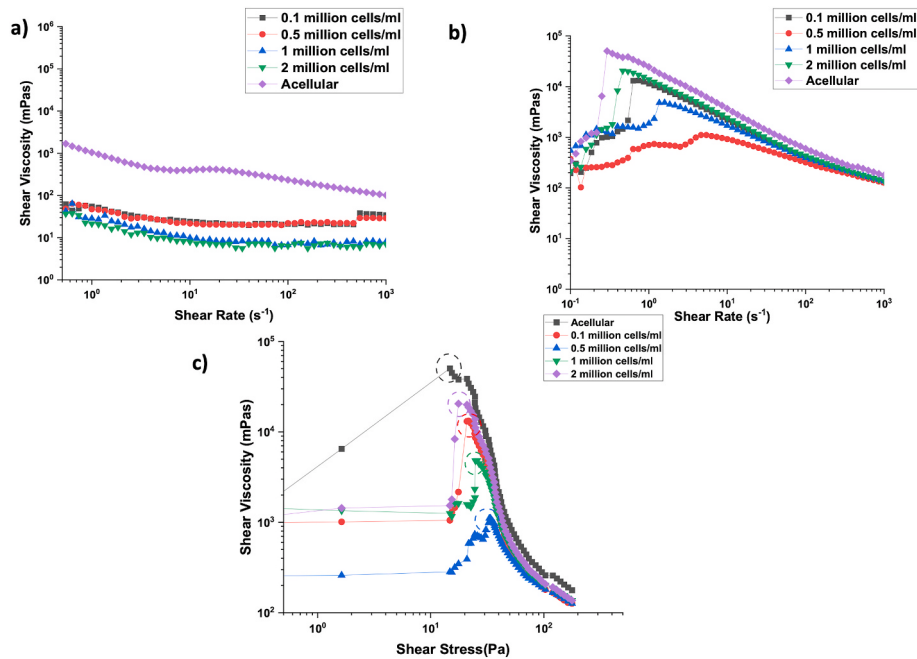


Fig. 2. Viscosity vs shear rate and viscosity vs shear stress analysis, a) Flow behaviour of acellular and cellular non-crosslinked 5SF-6G bioinks, b) Flow behaviour of acellular and cellular crosslinked 5SF-6G bioinks, c) Yield points of crosslinked cellular and acellular 5SF-6G bioinks.

bioinks. A shear-thinning behaviour i.e., decreased viscosity with increasing shear, was observed for all cell-laden bioinks and the acellular 5SF-6G control. Viscosity decreased with increasing cell concentration at all the shear rates. When comparing the decline in viscosity of acellular bioink with 0.1 million cells/ml at 200 s⁻¹, 88% drop was observed, while at 400 s⁻¹ shear rate, 85% drop was observed. On comparing cellular bioinks, the lowest range of viscosity values was found in 2 million cells/ml concentration, while the highest viscosities were observed in 0.1 million cells. However, using a crosslinker-free bioink for real-time applications will not be suitable due to the thermos-responsive characteristics of gelatine in the 5SF-6G bioink at higher temperatures (>30 °C) [46]. Thus, irreversible stability is imparted to the bioink using an enzymatic crosslinker mushroom tyrosinase. Fig. 2b represents the flow behaviour of both acellular and cell-laden bioinks cross-linked with 800 U mushroom tyrosinase enzyme. A catastrophic decrease in viscosity is observed in the case of all cell-laden bioinks similar to the acellular control group demonstrating a shear thinning behaviour. Moreover, a clear shift in the onset of viscosity drop is visible with the incorporation of cells in the 5SF-6G bioink compared to the acellular 5SF-6G hydrogel. This drop is due to the breakdown of the network structure where the hydrogel starts flowing, which is also called the yield point [47]. Das et al. reported that the applied shear rate in 250 µm diameter nozzle required for 3D bioprinting is in the range of 198.7–386.7 s⁻¹ [27]. Thus, the apparent viscosities of acellular and cellular bioinks at 200 and 400 s⁻¹ from the flow curve are reported in Table 3. The shear thinning behaviour of both cross-linked and non-crosslinked bioinks were quantified through power law equation,

Table 3

Viscosity table of acellular and cellular crosslinked 5SF-6G bioink at 3D bioprinting shear rates.

Sample ID	200 s ⁻¹	400 s ⁻¹
Acellular	364.14 ± 65.12 mPas	258.96 ± 31.98 mPas
0.1 million cells/ml	56.09 ± 8.43 mPas	79.96 ± 18.54 mPas
0.5 million cells/ml	228.31 ± 32.5 mPas	181.02 ± 22.93 mPas
1 million cells/ml	265.63 ± 89.74 mPas	201 ± 37.6 mPas
2 million cells/ml	280.05 ± 25.32 mPas	206.4 ± 27.31 mPas

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

Where K represents consistency index (initial viscosity), n represents power-law index or shear-thinning coefficient and η represents apparent viscosity. Table 2 illustrates the power law equations and their corresponding K and n values of respective cellular and acellular bioinks. The R² values of crosslinker-free bioinks were calculated much less than 1 (Data not shown) as compared to the cross-linked bioinks. Thus, enzymatic cross-linking of 5SF-6G bioinks resulted in a more consistent shear thinning nature of these hydrogels imparting additional mechanical stability compared to the non-crosslinked blends. The yield stress values evaluated from the viscosity vs. shear stress graph was found to decrease with increasing cell encapsulation (Fig. 2c). A possible reason could be due to the strain-softening effect induced by cell-matrix interaction.

3.3. Effect of cells on the modulus of silk fibroin-gelatin bioink

3.3.1. oscillatory amplitude sweep

For fabricating a stable 3D bioprinted construct, it necessary to study

Table 2

Power law equation for crosslinked cellular and acellular 5SF-6G bioink quantifying the shear thinning behavior.

Sample ID	Power law equation	R ² value of equation	K = consistency index (Pa s ⁿ)	n = power law index (dimensionless)	Yield Stress (Pa)
Acellular	$\eta = 24.5\gamma^{-0.0268}$	0.9945	24.5	0.9732	14.78
0.1 million cells/ml	$\eta = 11.4\gamma^{-0.0268}$	0.9936	11.4	0.9544	20.992
0.5 million cells/ml	$\eta = 7.03\gamma^{-0.0291}$	0.9979	7.03	0.9709	33.017
1 million cells/ml	$\eta = 18.7\gamma^{-0.0381}$	0.9934	18.7	0.9619	24.872
2 million cells/ml	$\eta = 13.86\gamma^{-0.046}$	0.9909	13.86	0.9540	17.586

the effect of various cell concentrations on the viscoelastic behaviour of bioink. Firstly, to demonstrate the efficiency of the enzymatic cross-linker as stimulus to mechanical stability of the bioink, an oscillatory amplitude sweep of both cross-linked and non-crosslinked bioinks was carried out. From Fig. 3, it is evident that the flow point in the case of non-crosslinked acellular bioink occurred at a much earlier strain % (101%) whereas in the case of cross-linked bioink, it was observed at 652% shear strain. Thus, a higher shear strain is required to dis-entangle the cross-linked polymeric chains and flow than the crosslinker-free 5SF-6G blend. This validates the need for enzymatic cross-linking in fabricating mechanical stable 3D constructs.

Amplitude strain sweep is generally performed to assess the shear strain range at which storage modulus remains constant [48]. This linear viscoelastic range (LVR) is utilized for further frequency sweep analysis. An oscillatory amplitude sweep analysis was performed for all the enzymatically cross-linked acellular and cellular bioinks (0.1, 0.5, 1 and 2 million cells/ml) to evaluate the LVR and their respective flow points. It is observed from Fig. 4a that a decrease in storage modulus in cell-laden bioinks is observed compared to the acellular bioink depicting the possible role of cell density in affecting the modulus properties of the bioink. Table 4 demonstrates the dynamic storage modulus values of acellular and cell-laden 5SF-6G bioinks. This is further validated by the occurrence of an early flow point in the case of cellular bioinks compared to that of an acellular bioink flow point, which occurred at 652% shear strain (Fig. 4b). The characteristic linear viscoelastic region in all the bioinks was considered around 1% shear strain for further experimentation to minimize sample fracture.

3.3.2. oscillatory frequency sweep

To analyse the development in the degree of softness of the hydrogel, a frequency sweep of both cellular and acellular bioinks was carried out. Frequency sweep can also determine the appropriate frequency value for time sweep [44]. A substantial drop in storage modulus was observed with increasing cell concentrations in all cell-laden bioinks compared to the acellular control (Fig. 5a). At an angular frequency of 100 rad/s, storage moduli of acellular, 0.1, 0.5, 1 and 2 million cells/ml cellular concentration containing bioinks were 52 Pa, 49 Pa, 16 Pa, 8.8 Pa and 3.2 Pa. Such sheer drop in storage modulus (Fig. 5b) could be due to incorporating a cellular concentration gradient in the respective bioinks that interfered with the mushroom tyrosinase activity during the process of enzymatic cross-linking in 5SF-6G bioinks as previously described [49]. Additionally, encapsulation of cells in the bioink also contributed to the increasing the softness of the gel due to the frequency-dependent

modulus properties (G' and G'') for all the cell-laden bioinks. The loss factor ($\tan \delta$) or the ratio of loss modulus (G'') to storage modulus (G') was evaluated the intrinsic behaviour of the cell-laden bioinks [46]. A loss factor less than 1 signifies the gel-like behaviour of the bioink, whereas a loss factor greater than 1 signifies the viscous nature of the bioink [47]. From Fig. 5c, it can be visualized that the loss factor of the cell-laden bioinks increased with increasing cell concentration, with a maximum value of 0.538 for 2 million cells/ml. This can be attributed to the fact that encapsulation of cells might disrupt the cross-linking mechanism of mushroom tyrosinase within the bioink that influences the strength of the respective hydrogels. The encapsulation of the cell can also alter the rigidity of the hydrogel and change the gelation behaviour of the respective bioinks [50].

3.4. Effect of cells on the gelation kinetics of the silk fibroin-gelatin bioink

An oscillatory time sweep experiment was performed to understand how cells alter the gelation behaviour (Gelation time) of the bioink. The gelation occurs due to the transformation of the liquid phase of hydrogel to a three-dimensional structure formed from strong covalent bonds [51]. The time point at which the storage modulus becomes equal to the loss modulus is called the gelation point/crossover point (T_g). This gelation time (T_g) depends on the shear strain % and frequency parameters given during the test [52]. It is clearly demonstrated in Fig. 6 that the gelation times of cell-laden bioinks increased with increasing cell concentration, with the acellular bioink having the lowest gelation time of 1.17 min, whereas the highest gelation time of 7.67 min is observed in the case of 2 million cells/ml. This increase in T_g can be attributed to the increasing concentration of cells inhibiting the underlying cross-linking mechanism and delaying the formation of an enzymatically cross-linked hydrogel network. Post gelation point, increased storage modulus has been observed for all the cross-linked acellular and cell-laden bioinks depicting efficient gelation.

3.5. Printability of acellular and cell-laden bioinks

The printability of the acellular and cell-laden bioinks (1 million and 2 million cells/ml) was evaluated as a shape fidelity function by calculating the spreading ratio [53]. The spreading ratio can be defined as the ratio of the extruded filament diameter to the nozzle's diameter. A lower spreading ratio is generally expected to develop cell-encapsulated 3D structures with high precision as observed in case of GelMA when compared with alginate and agarose mixed with BMSCs by Daly et al. [54]. The spreading ratio of acellular 5SF-6G bioink was calculated to be 2.39 ± 0.021 , whereas, for the 5SF-6G bioink containing 1 million cells/ml and 2 million cells/ml, the spreading ratio was around 3.28 ± 0.021 and 3.67 ± 0.031 , respectively (Fig. 7a). The printability index was further calculated from the geometric accuracy of the axial pore in the XY plane in exhibiting a perfect square structure. Printability index (P_r) = 1 corresponds to perfect square geometry, whereas $P_r > 1$ or < 1 corresponds to irregular or circular geometry [53]. In our present study, $P_r = 1$ has been achieved for acellular, and 1 million cells/ml encapsulated bioink indicating optimum gelation and viscosity. For 2 million cells/ml, laden 5SF-6G bioink depicted a $P_r < 1$ due to decreased viscosity and mechanical stability (G') (Fig. 7b).

3.6. Viability of cells pre- and post 3D bioprinting

From the flow cytometry analysis (Fig. 8), it is clearly visible that the cells within the 5SF-6G bioink before 3D bioprinting demonstrated a cell viability of 90.24% (PI uptake: 9.76%) whereas it slightly decreased to 70.43% post-printing (PI uptake: 29.57%). Such slight decrease in cell viability post extrusion can be attributed to the cylindrical nozzle shape exposing the cells to enhanced shear stress throughout the length of the nozzle during extrusion.

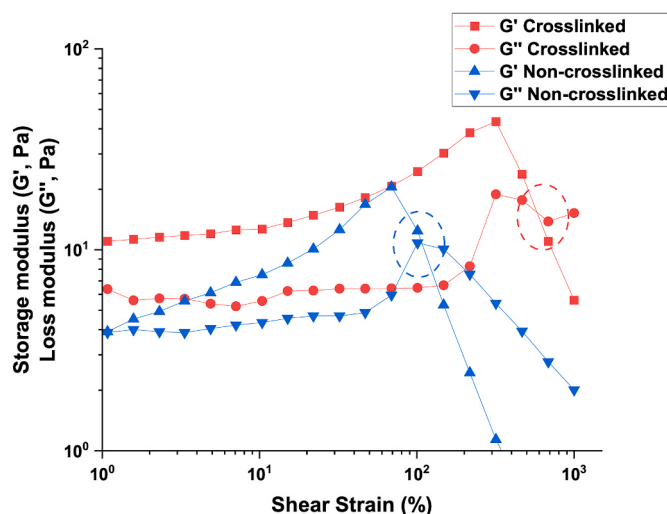


Fig. 3. Oscillatory amplitude sweep analysis crosslinked and non-crosslinked acellular 5SF-6G bioinks.

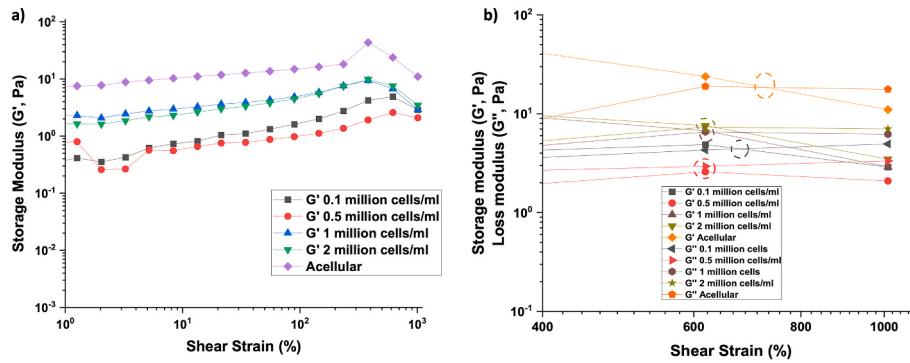


Fig. 4. Oscillatory amplitude sweep analysis, a) Storage modulus of enzymatically crosslinked cell encapsulated and acellular 5SF-6G bioinks, b) Flow point analysis of crosslinked 5SF-6G bioinks.

Table 4

Storage modulus table of crosslinked cellular and acellular 5SF-6G bioink from oscillatory amplitude sweep.

Sample ID	10% shear strain	100% shear strain
Acellular	18.28 ± 1.45 Pa	30.63 ± 7.52 Pa
0.1 million cells/ml	0.82 ± 0.12 Pa	1.97 ± 0.51 Pa
0.5 million cells/ml	0.65 ± 0.211 Pa	1.16 ± 0.192 Pa
1 million cells/ml	3.24 ± 0.976 Pa	5.83 ± 0.992 Pa
2 million cells/ml	2.64 ± 0.76 Pa	5.85 ± 1.34 Pa

4. Discussion

Designing an appropriate bioink with optimum rheological, mechanical, and biological properties is crucial in 3D Bioprinting [55,56]. Rheological characterizations of any biomaterial are mainly carried out to determine the viscosity, yield stress, viscoelastic behaviour and shear thinning nature of the biomaterial. These parameters are further utilized to ultimately evaluate the cell encapsulation potential, extrusion velocity, nozzle speed and printing pressure required for smooth extrusion through the orifice with minimum exposure of shear stress [5,53]. An

extensive review revealing the critical prerequisites for 3D bioprinting with silk biomaterial by Chawla et al. mentioned shear-thinning nature as an essential rheological parameter to ensure smooth printability and enhanced print fidelity post printing [2]. However, standalone regenerated silk fibroin solution depicted Newtonian flow behaviour [57], but the addition of gelatin to the silk fibroin biomaterial induced shear thinning characteristics in the blend [27]. Such change in behaviour is assumed to be the ionic interaction at physiological pH due to differences in isoelectric points resulting in formation of a semi-interpenetrating network [58]. To counteract the challenge pertaining to the loss of structural stability of the SF-G hydrogel during cell culture (37°C) due to the thermo-responsive behaviour of gelatin, an additional irreversible enzymatic cross-linking of silk fibroin-gelatin blend is performed using mushroom tyrosinase [21]. The underlying mechanism of the enzymatic crosslinking lies in the oxidation of tyrosine moieties of silk fibroin and gelatin by the tyrosinase enzyme and further condensation of the developed quinone residues or nucleophilic substitution to form an irreversible bond [59].

Previous investigations from our lab extensively studied the rheological properties of standalone silk fibroin as well as SF-G blend for a wide array of concentrations and ratios. A study by Das et al. using

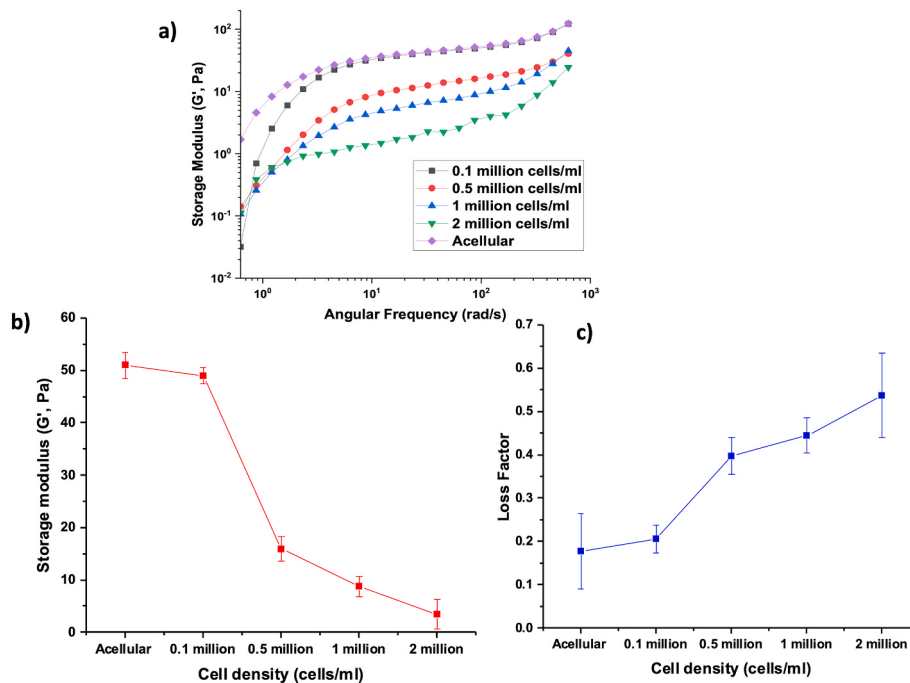


Fig. 5. Oscillatory frequency sweep analysis, a) Storage modulus of crosslinked cell-laden and acellular 5SF-6G bioinks, b) Storage modulus of crosslinked 5SF-6G bioinks with increasing cell concentration at 100 Hz, c) Loss Factor of crosslinked 5SF-6G bioinks with increasing cell concentration at 1 Hz.

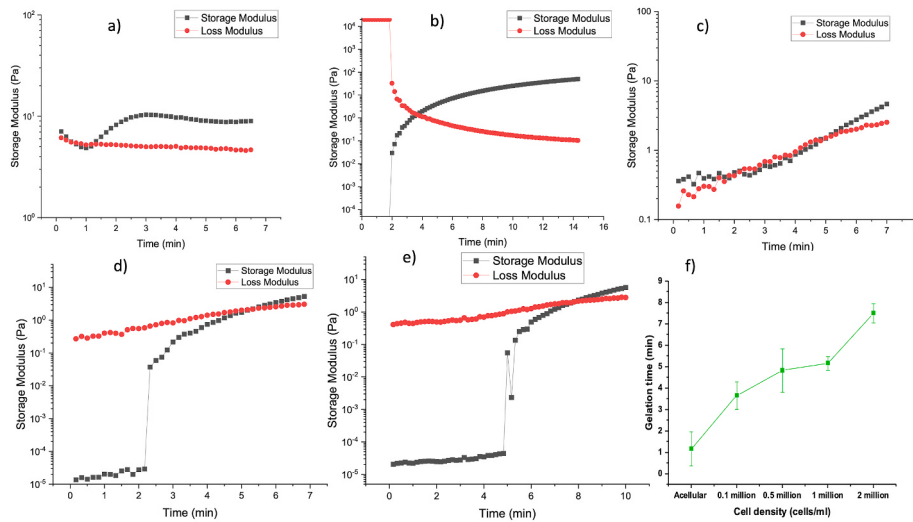


Fig. 6. G' and G'' as a function of time to study the gelation time of a) acellular, b) 0.1 million cells/ml, c) 0.5 million cells/ml, d) 1 million cells/ml, e) 2 million cells/ml 5SF-6G bioink, f) Gelation time evaluation of 5SF-6G crosslinked bioinks with increasing cell concentration.

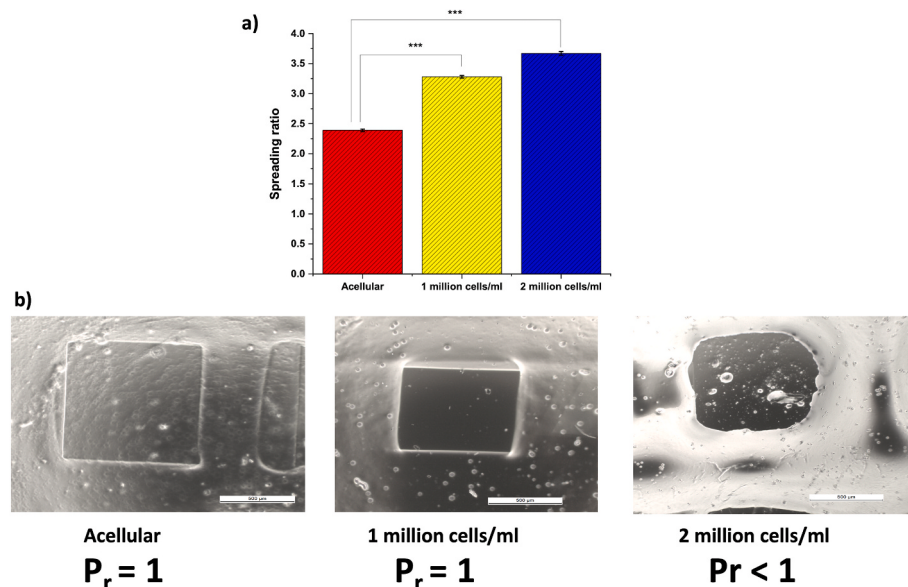


Fig. 7. Printability assessment of acellular and cell-laden crosslinked 5SF-6G bioinks, a) Spreading ratio of acellular and cellular crosslinked 5SF-6G bioinks, (***) $p < 0.001$ b) Printability index of acellular and cellular crosslinked 5SF-6G bioinks.

different concentrations of silk fibroin (SF) and SF-G blend (crosslinker free) revealed a characteristic shear thinning behaviour of standalone SF % up to 20% whereas a consistent decrease in apparent viscosity with increasing shear rate was observed for all combinations of SF-G blend [26]. A comparative study of the rheological property of the bioinks cross-linked via sonication and mushroom tyrosinase respectively, also demonstrated a more typical shear thinning characteristics than previously described crosslinker-free SF-G blends, necessitating the role of cross-linking in inducing irreversible mechanical stability in the bioink [27]. Although all these results suggested the plausible shear thinning behaviour of the SF-G blend required for 3D Bioprinting, it does not recapitulate the real-time rheological profile of the bioink during 3D bioprinting by neglecting the presence of cells in it. Our present study assessed the flow behaviour of both the enzymatically cross-linked and non-crosslinked 5SF-6G bioinks comprising four different cell densities (0.1, 0.5, 1 and 2 million cells/ml) and compared them with an acellular control. The enzymatic crosslinking of 5SF-6G bioink via mushroom tyrosinase enzyme was confirmed using ATR FTIR analysis depicting

higher intensity peaks in the desired regions than the non-crosslinked group (Fig. 1). The non-crosslinked acellular bioink displayed a shear thinning behaviour with an R^2 value of 0.9732, whereas all the cell-laden bioinks displayed an inconsistent shear thinning behaviour for all cell concentrations with an R^2 value $\ll 1$ (Table 4). A notable fact from Fig. 2a is the drop in initial viscosity with increasing cellular concentrations in non-crosslinked bioinks. We postulated that the absence of any additional covalent crosslinker other than already present physical and ionic cross-links led to the disruption of the hydrogel network by cells. The cross-linked group of bioinks (both cell-laden and acellular) demonstrated a sharp decrease in viscosity with increasing shear rates as evident from Fig. 2b. All the sample groups (acellular and cell-laden) from the flow curve depicted a confidence fit of 0.99 when fitted in Ostwald-de Waele Power law model. The cell-laden bioinks displayed a power law index (n -value) less than 1 signifying a shear thinning bioink with consistency index value of 1 million cells/ml (18.7 Pas) closer to the acellular 5SF-6G blend (24.5 Pas). Such decrease in viscosity with increasing cell concentration is in line with the study by R.

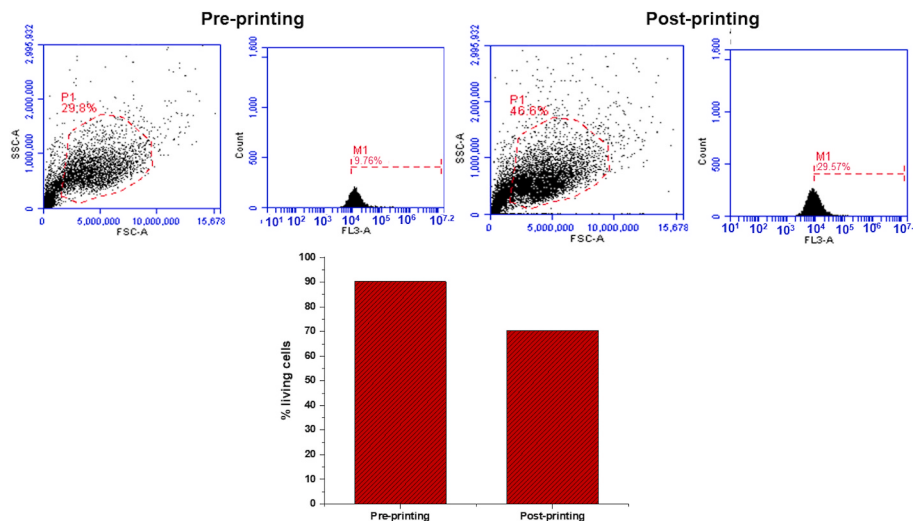


Fig. 8. Flow cytometry analysis depicting cell viability before and after 3D bioprinting.

Schwartz et al. The decreasing viscosity with increasing cell concentration is explained as a function of a decrease in cell-hydrogel space that interferes with the rheological evaluation under any physical deformation [37]. However, a contradictory result has been published by Xu et al., where increased viscosity has been reported with increasing cell concentration at a fixed shear rate [38]. Such contrasting findings concerning our present study can be attributed to the dilute nature of their Alginate bioink compared to our concentrated SF-G bioink and the nature of the cross-linking method (chemical cross-linking for alginate and enzymatic cross-linking for SF-G). The yield stress of the bioinks also exhibited a decreasing trend with increasing cell concentration (Fig. 2c), validating our viscosity vs. shear rate data. Furthermore, Schwartz et al. reported a similar decline in yield stress with increasing cell densities in the bioink due to the cells causing blockage of inter-chain interactions within the polymeric blend [37].

The viscoelastic properties of the bioink also play a vital role in 3D bioprinting as it is a crucial determining factor in the amount of shear stress on the encapsulated cells during extrusion [5]. An oscillatory amplitude/strain sweep experiment was conducted for non-crosslinked and enzymatically cross-linked acellular bioink to probe the role of mushroom tyrosinase mediated cross-linking as a significant regulator of modulus properties of the bioinks. From Fig. 3a, the storage modulus of the cross-linked bioink at 1% strain is higher than the storage modulus of the non-crosslinked bioink at 1% strain, indicating an increased mechanical stability of the bioink with enzymatic cross-linking. This is following the results published by Das et al. in which an elevated storage modulus (530,000 Pa) of tyrosinase cross-linked 8SF-15G blend (SF:G ratio- 0.53) was observed compared to the storage modulus (7.71 Pa) of crosslinker-free 5SF-10G blend (SF:G ratio-0.5) [26] at 1% strain. Moreover, the flow point, defined as the critical shear strain at which dynamic storage modulus (G') equals the dynamic loss modulus (G'') occurred at a much later shear strain for cross-linked bioink compared to the crosslinker-free bioink. Thus, it can be inferred that a higher shear strain is required to rupture the polymer entanglements for an enzymatically cross-linked 5SF-6G blend. Beyond the flow point, the un-entangled polymeric chains align themselves in the flow direction resulting in the disruption of the semi inter-penetrating network of the 5SF-6G hydrogel [58]. The oscillatory amplitude sweeps of all the cross-linked cell suspension encapsulated 5SF-6G bioinks demonstrated a decrease in storage modulus with increasing cellular concentrations in the bioink at a fixed shear strain of 1%. The 5SF-6G bioink encapsulated with 1 million cells/ml portrayed a storage modulus closer to the acellular bioink (Fig. 4a), rendering comparatively higher shape fidelity and structural stability post-printing. This is in line with the findings by Zhao

et al. that incorporating 1 million cells/ml A549 cell line into gelatin-alginate hydrogel slightly decreased the storage modulus of the blend as compared to the acellular hydrogel [35]. A similar trend is also elucidated by Biillet et al. [36], and Diamantides et al. [34] in HepG2 encapsulated gelatin methacrylamide and human chondrocytes embedded collagen bioinks respectively. Another interesting finding from the oscillatory amplitude sweep is the early occurrence of flow point in the cell-laden bioinks compared to the acellular 5SF-6G biomaterial (Fig. 4b). Thus, increasing the cell concentrations in the bioink might hinder the enzymatic crosslinking of the composite hydrogel leading to weak gel formation. As a result, a very low shear strain is sufficient to shatter the hydrogel network. A shear strain of 1% was used for further oscillatory frequency sweep analysis to avoid sample fracture during the experimental procedure. Fig. 5b portrays the decreasing trend of storage modulus with increasing concentration of cells in the bioink at an angular frequency of 100 rad s^{-1} with 2 million cells/ml containing 5SF-6G bioink having the lowest G' (3.2 Pa) and acellular bioink possessing the highest G' (52 Pa). A similar trend is also observed in our gelation kinetics study, where an increase in gelation time was observed with an increase in the concentration of cell suspension from 0.1 to 2 million cells/ml (Fig. 6f). A clear justification for the above observation was given by Schwab et al. that the incorporated cells might interfere with the enzymatic crosslinking of the bioink by masking the active sites of the hydrogel or capturing the small molecule required for crosslinking initiation rendering it a weak gel character [53]. This behaviour was further observed by Petta et al. in their horseradish peroxidase (HRP) crosslinked hyaluronic acid (HA) hydrogel encapsulated with 1 million and 5 million cells/ml [61]. Moreover, from Fig. 5a, it is evident that the storage modulus (G') of both the crosslinked acellular and cellular bioinks illustrated a linear dependency with angular frequency (ω). Such dependency of G' on ω is a characteristic feature of a weak gel system, as explained by Marapureddy et al. [60].

The main objective of the above rheological analysis of cell-laden bioinks was to identify the optimum cell concentration required for achieving high print fidelity, and cell viability during and post 3D bioprinting. Thus, a preliminary printability analysis was carried out for the acellular and cell-laden 5SF-6G blends. Here, cell densities of 1 million cells/ml and 2 million cells/ml were considered as already described in section 2.5 that low cell concentrations in the bioink will not be a suitable approach for developing tissue-engineered constructs. Moreover, the storage modulus values of 1 million cells/ml and 2 million cells/ml were closer to the acellular 5SF-6G blend, making it suitable for 3D bioprinting with high shape fidelity (Fig. 5a). Additionally, the loss

factor values calculated from the frequency sweep analysis for 1 million (0.445) and 2 million cells/ml (0.538) fall under the printability window as elucidated by Petta et al. making them the perfect suit for printability experiments [61]. Also, the loss factor of acellular bioink was evaluated as 0.178, which falls in the non-printable window. As a result, a high pressure of 30 PSI was required for smooth extrusion of the hydrogel as mentioned in Table 1. The calculated spreading ratio of 1 million cells/ml was 3.28 ± 0.021 , close to the spreading ratio of acellular bioink (2.39 ± 0.021), signifying adequate shape fidelity post bioprinting. Ideally, ink spreading ratio should be equal to 1 i.e., the diameter of the extruded filament should be equal to the diameter of the nozzle. In our study, ink spreading occurred for all our acellular and cell-laden bioinks possible due to lower surface tension and higher gravitational pull compared to the surface energy of the deposited surface [62]. The printability index (P_r) evaluation of the 3D bioprinted constructs revealed a $P_r = 1$ for the acellular and 1 million cells/ml encapsulated 5SF-6G bioink indicating the formation of an accurate square geometry compared to the 5SF-6G bioink embedded with 2 million cells/ml cell density. Such unit printability index for acellular and 1 million cells/ml encapsulated 5SF-6G bioink can be attributed to optimum gelation and viscosity as evident from the storage modulus (G') values in amplitude sweep and viscosity values at the extruding shear rate through a 230 μm (Table-2). However, the 5SF-6G blend encapsulated with 2 million cells/ml displayed a P_r value of less than 1, which is the direct result of improper gelation and decreased viscosity due to hindering of the enzymatic crosslinking by the increasing cell concentrations as described above. The cell viability analysis was also carried out using flow cytometry with 1 million cells/ml 5SF-6G group before and after 3D bioprinting. From the plots, it was observed that an approximately 70% cells were viable post-extrusion in the 3D bioprinted constructs than compared to the 90% cell viability for cells in 5SF-6G bioink before printing (Fig. 8). Such decrease can be attributed to the increased shear stress in the cylindrical nozzle during extrusion as well as during protease digestion step for cell extraction that might interfere with the overall cell viability in the 3D printed constructs [63].

Cells expressed a wide range of surface receptor proteins to communicate with their respective extracellular matrix domains through a series of signalling cascades among which integrins attain a pivotal role [31]. The interaction of the cell adhesion motifs of the surrounding hydrogel with the cell surface integrins induces a conformational change that allows its linkage to the actin cytoskeleton [33]. The integrin-actin linkage is mediated by a plethora of associated proteins (talin, vinculin, paxillin) and recruitment of focal adhesion kinases that ultimately governs the actin reorganization and overall cellular biomechanics [32]. Yadav et al. in their research also reported the expression of receptor protein CD-29 (99.87%) on TVA-BMSC cell surface determined through flow cytometry analysis [64]. This CD-29 or the β -subunit of integrins portray an essential role in regulating the cell-ECM interactions [65] and thus, altering the overall mechanics of the hydrogel. Thus, it can be rightly inferred from our findings that cells not only interfere with the 3D inter-penetrating network formation by hydrogel elements via hindrance in crosslinking process, but also its intrinsic mechanical properties that plays a vital role in cell-mediated alteration of rheological characteristics of a hydrogel.

5. Conclusions

A comprehensive study has been carried out to investigate the effect of TVA-BMSCs at different cell densities (0.1 million cells/ml, 0.5 million cells/ml, 1 million cells/ml, and 2 million cells/ml) on the rheological properties of the enzymatically crosslinked and non-crosslinked silk fibroin-gelatin (5SF-6G) bioink. From our findings, it is evident that non-crosslinked bioink displayed an inconsistent shear-thinning profile and low storage modulus (G') as compared to the mushroom tyrosinase crosslinked 5SF-6G bioink necessitating the requirement of enzymatic crosslinking for further assessments. The

rheological characterization of the cell-laden bioinks demonstrated that the incorporation of cells decreased the shear viscosity, storage modulus and loss factor ($\tan \delta$) while increasing the gelation time, exhibiting the typical characteristics of a weak gel system. The printability analysis also illustrated that high cell density (2 million cells/ml) in the bioink made it challenging to fabricate a perfect square geometry, whereas 1 million cells/ml exhibited a unit printability index with comparatively lesser ink spreading post extrusion. Thus, encapsulating 1 million cells/ml in 5SF-6G portrayed optimum rheological characteristics and printability necessary for fabricating structurally stable 3D constructs with increased cell viability, proliferation rate and differentiation potential. A practical futuristic approach would be to analyse the effect of different cell types on the rheological profile and printability of the hydrogel. Such approach would allow us to gain a more detailed insight into the underlying mechanism and related protein counterparts governing the cell-matrix interaction that ultimately alters the rheology of the bioink during microextrusion-based 3D Bioprinting.

CRedit authorship contribution statement

Nilotpal Majumder: Conceptualization, Methodology, Investigation, Writing – original draft, Data curation, Preparation of data, Writing – review & editing. **Aditya Mishra:** Methodology, Investigation. **Sourabh Ghosh:** Conceptualization, Resources, Supervision, Funding acquisition, Validation, Writing – review & editing.

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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