Supporting Information

3D Bioprinting using Cross-Linker Free Silk-Gelatin Bioink for Cartilage Tissue Engineering

*Yogendra Pratap Singh[1], Ashutosh Bandyopadhyay[1], Biman B. Mandal[1,2]**

[1]Biomaterial and Tissue Engineering Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati – 781039, Assam, India.

[2]Centre for Nanotechnology, Indian Institute of Technology Guwahati, Guwahati – 781039, Assam, India.

***Corresponding author:** Biman B. Mandal

E-mail: biman.mandal@iitg.ac.in, mandal.biman@gmail.com

Tel: +91-361-258-2225 Fax: +91-361-258-2249

1. Table S1. Details of primers used for real-time PCR study.

Gene	Sequence	Accession No.
pAggrecan	F 5′-CCCAACCAGCCTGACAACTT-3′	NM_001164652.1
	R 5′-CCTTCTCGTGCCAGATCATCA-3′	
pSox-9	F 5′-TTCCGCGACGTGGACAT-3′	NM_213843.1
	R 5′-GGCGGCAGGTACTGGTCAAACTC-3′	
pCollagen II	F 5′-CAGGTGAAGGTGGGAAACCA-3′	AF201724.1
	R 5′-ACCCACGAGGCCAGGA-3′	
pCollagen X	F 5′-ACGGGCAACAGCACTATGACC-3′	NM_001005153.1
	R 5′-GCACTCCCTGAAGCCTGATCC-3′	
pGAPDH	F: 5′-TCGGAGTGAACGGATTTGG-3′	NM_001206359.1
	R: 5′-CCAGAGTTAAAAGCAGCCCT-3′	

2. Rheological properties of the silk fibroin-blend without gelatin

Rheological characteristics of the silk blend without gelatin was carried out under the same conditions as that of the optimized silk-gelatin ink. **Figure S1A** depicts the complex viscosity of the ink with a constant viscosity of ~3 Pa.s till about 42 °C and it gradually increases after that. This is unlike the silk-gelatin ink where a temperature window of 25-35°C gives us a suitable viscosity for printing. The modulus showed a ~5 folds decrease in case of the silk blend without gelatin within the printing range of 20-25℃ (**Figure S1B**). Gelatin acts as a prominent bulking agent that helps in maintaining the printing fidelity of the ink. This factor is further established by the amplitude sweep (**Figure S1C**). Lower storage modulus and an earlier crossover point of the silk blend without gelatin was observed as compared to the optimized ink with gelatin. Moreover, frequency sweep (**Figure S1D**) comparison of the silk blend with and without gelatin clearly depicts the weakened strength of the silk blend gel (without gelatin) within the respective linear viscoelastic range, showing its unsuitability for 3D bioprinting application.

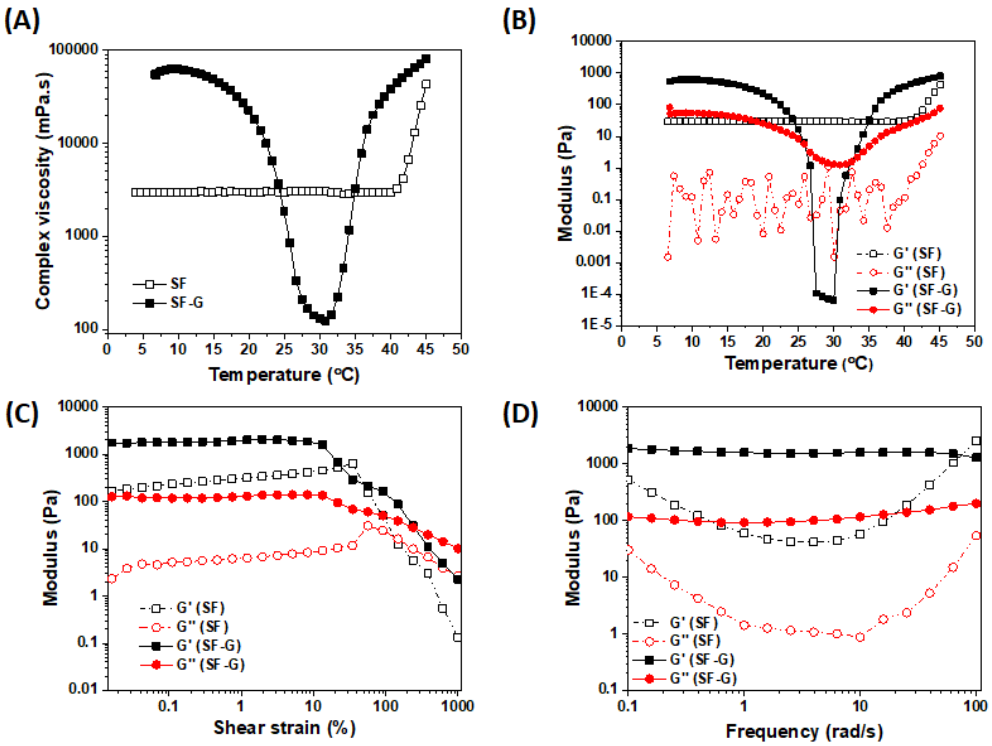

Figure S1. *Rheological assessment- (A) Complex viscosity of the ink from 5 ℃-45 ℃, (B) temperature sweep profile indicating the modulus over the temperature range. (C) Amplitude sweep, and (D) frequency sweep of the ink (SF-silk fibroin without gelatin and SF-G- silk fibroin with gelatin) performed at 25 ℃ (G' represents storage modulus, and G'' represents loss modulus).*

3. Print fidelity and geometry of the printed construct

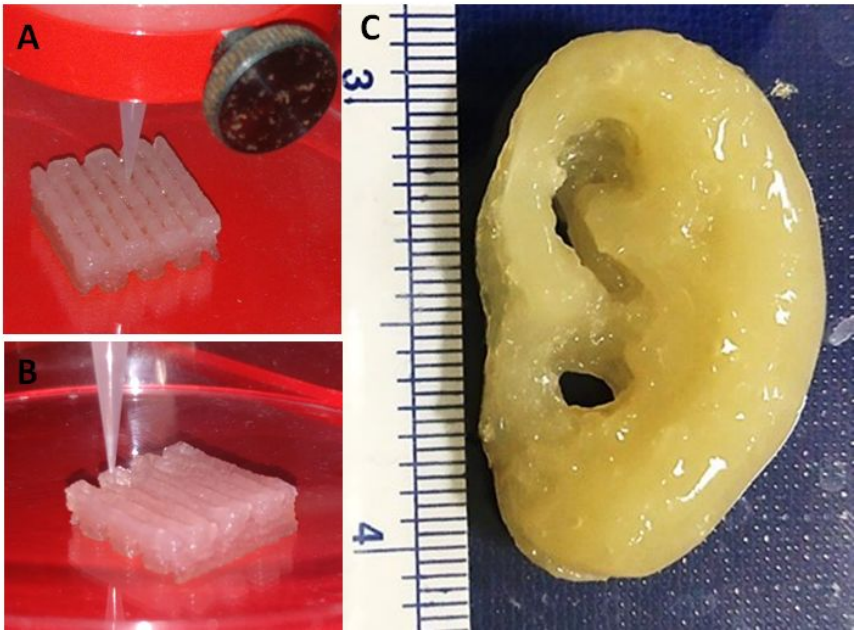

Figure S2. *(A-B) Gross morphology of the printed grid-like structure and (C) anatomically similar human ear using silk-gelatin blend ink.*

4. Stability of the construct in PBS

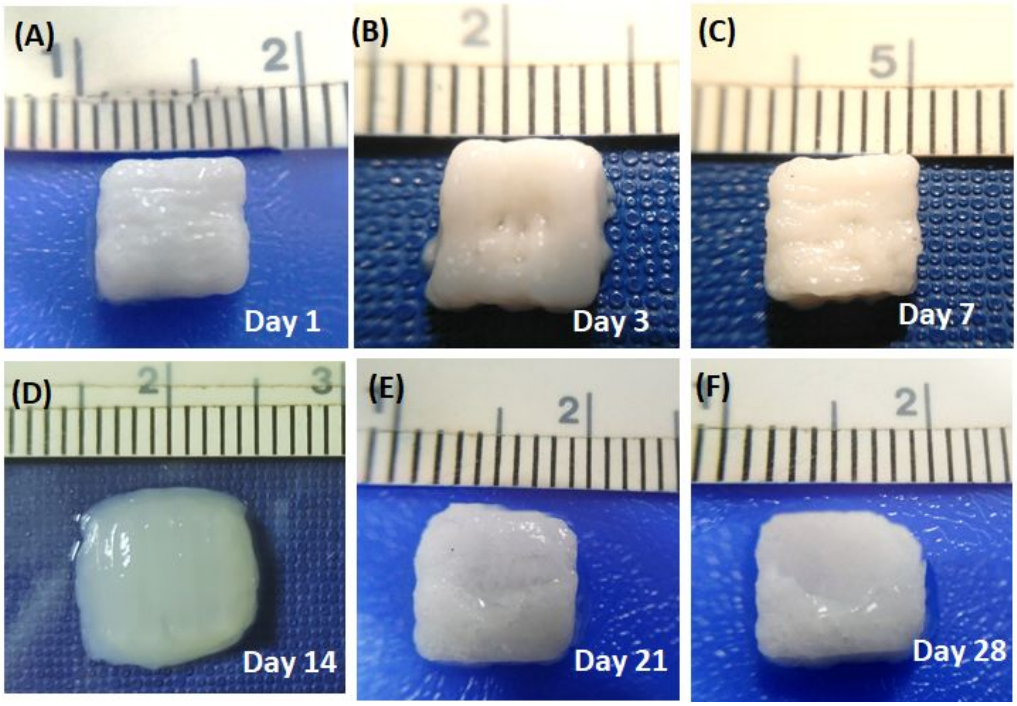

Figure S3. *Gross morphology showing the stability of construct in PBS (pH 7.4) at 37 °C after (A) 1 day, (B) 3 days, (C) 7 days, (D) 14 days, (E) 21 days, and (F) 28 days.*

5. Cell viability and *in vitro* histology

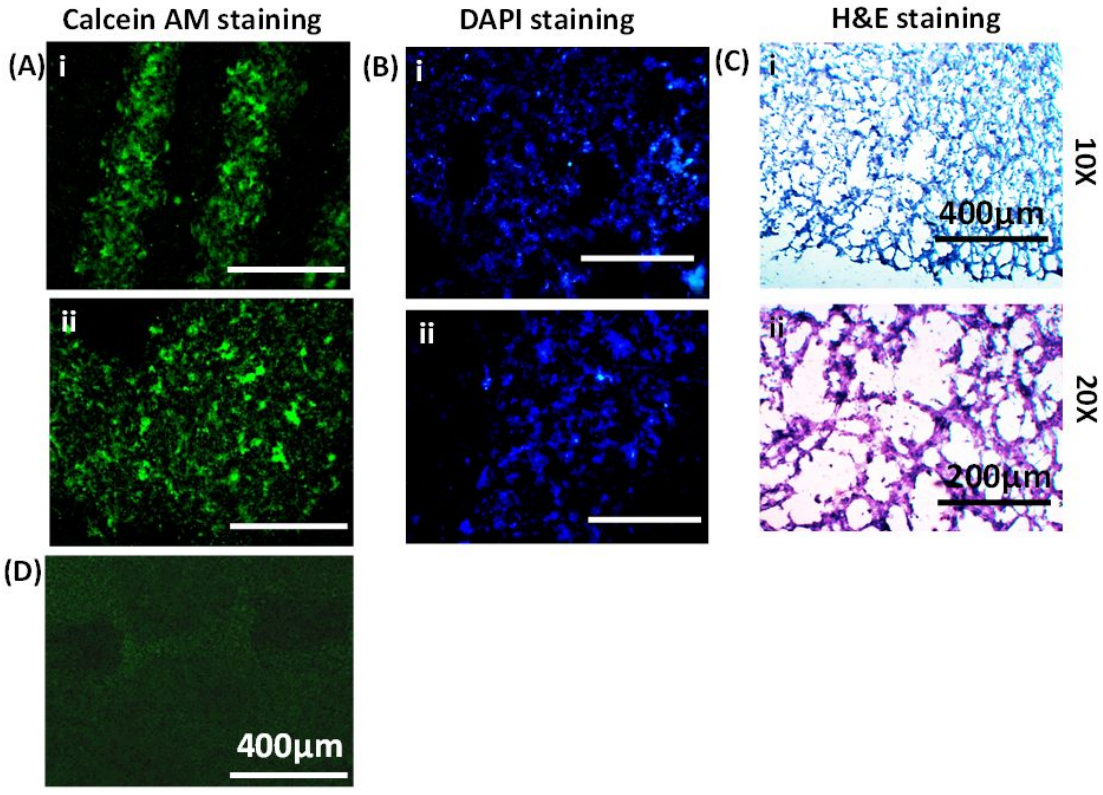

Figure S4. *(A) Calcien AM stained images of the chondrocytes laden bioprinted construct after 14 days of culture. Histological assessment of in vitro cultured bioprinted constructs post 14 days of culture (B) H&E stained section of the construct and (C) DAPI labelled bright blue cells. (D) Representative image of a bioprinted construct without cells that shows the background auto-fluorescence of silk fibroin matrix. Scale bar for (i) is 400 µm, for (ii) is 200 µm and (D) is 400 µm.*

6. *In vivo* biocompatibility - Histology

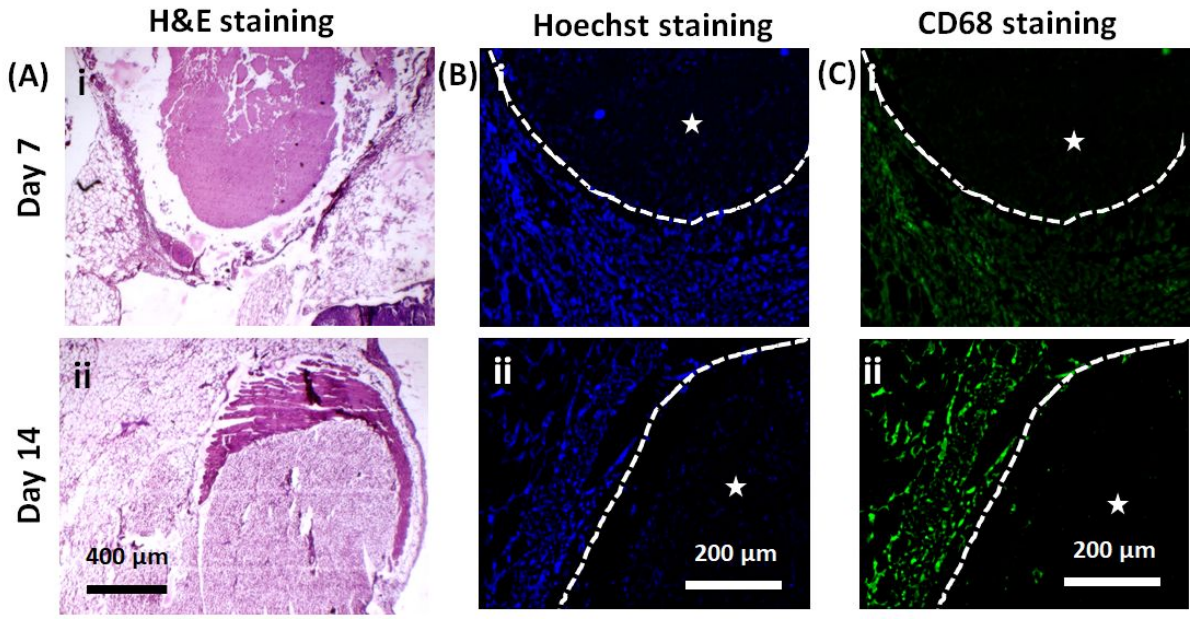

Figure S5. *Histological assessment of subcutaneously implanted ink in mice. (A) H&E stained sections retrieved after (i) 7 days and (ii) 14 days of implantation. Representative images showing (B) Hoechst stained nuclei of cells and (C) CD68 antibody staining for macrophages on (i) day 7 and (ii) day 14. Star represents the implanted ink. Scale bar for (A) is 400µm and for (B&C) is 200µm.*

7. *In vivo* biocompatibility – macroscopic image of the retrieved implant

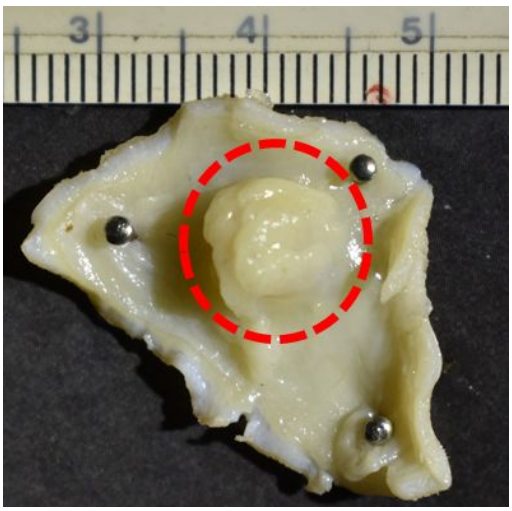

Figure S6. *Representative macroscopic image of the retrieved tissue containing encapsulated ink hydrogel implanted subcutaneously in mice for 14 days.*

8. Release of gelatin from the ink hydrogel

Method: The release of gelatin from the silk-gelatin ink was assessed in PBS at 37 °C using Sirius red dye according to a modified previously established protocol mentioned in Materials and Methods section 2.5.3. Briefly, the leachate at different time points was collected and dried at 37 °C for 24 h. To it, 100 µL of Sirius red dye solution (prepared in picric acid-saturated solution to a final concentration of 1 mg mL^{-1}) was added and incubated for 1 h with mild shaking. The samples were washed three times using 0.01 N HCl and the dye sample complex was resolved using 0.1 N NaOH. The absorbance was recorded at 550 nm. Inks without gelatin (only silk fibroin blend) were used as negative control.

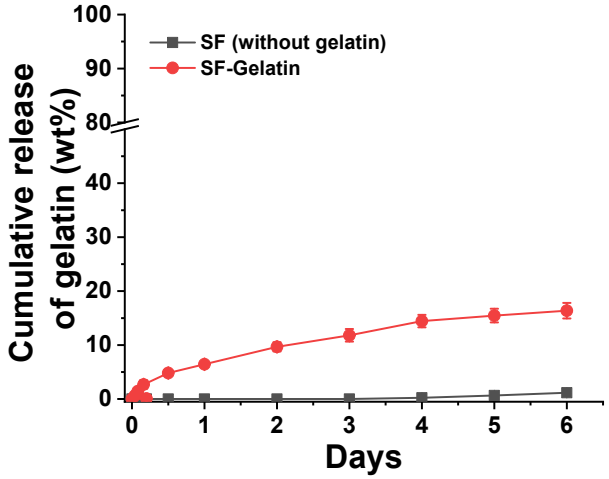

Figure S7. *The cumulative release of gelatin from the silk-gelatin inks performed at 37 °C. Inks without gelatin (only silk fibroin) were used as negative control.*

Result: In order to see the stability of the inks, they were incubated in PBS at 37 °C and the leachate was analysed for the release of gelatin for a time period of 6 days. The results show a slow release of gelatin from the inks. In 6 days a maximum of 16 wt% of the total entangled gelatin was found to leach from the ink indicating its stability (**Figure S7**). As a negative control, silk fibroin blend of *B. mori* and *P. ricini* containing no gelatin was used. The controls showed no interference with the gelatin release assay indicating the specificity of the assay for detection of gelatin.