Supporting Information: Engineering DN hydrogels from regenerated silk fibroin and poly(N-vinylcaprolactam)

Jasmin L. Whittaker[a], Naba K. Dutta[a,b], Andrew Zannettino[c], Namita Roy Choudhury[a,b]

[a]Future Industries Institute, Mawson Lakes Campus, University of South Australia, Mawson Lakes, Adelaide, SA 5095, Australia

[b] School of Chemical Engineering, University of Adelaide, Adelaide, SA 5005, Australia

[C]Myeloma Research Laboratory, School of Medical Sciences, University of Adelaide, Adelaide, SA 5005, Australia

1. NMR analysis of PVCL

^{1}H NMR spectra of VCL and PVCL were obtained from 300 MHz Bruker Avance spectrometer operating at 300.138 MHz. The solvent used was deuterated chloroform ($CDCl_3$). The peak assignments for the ^{1}H-NMR spectra of VCL and PVCL are shown in Table 4. All protons are accounted for and the disappearance of the signal for the vinyl proton indicated that there was no residual monomer in the polymer sample. The results agree with reported literature values for this material.[1, 2]

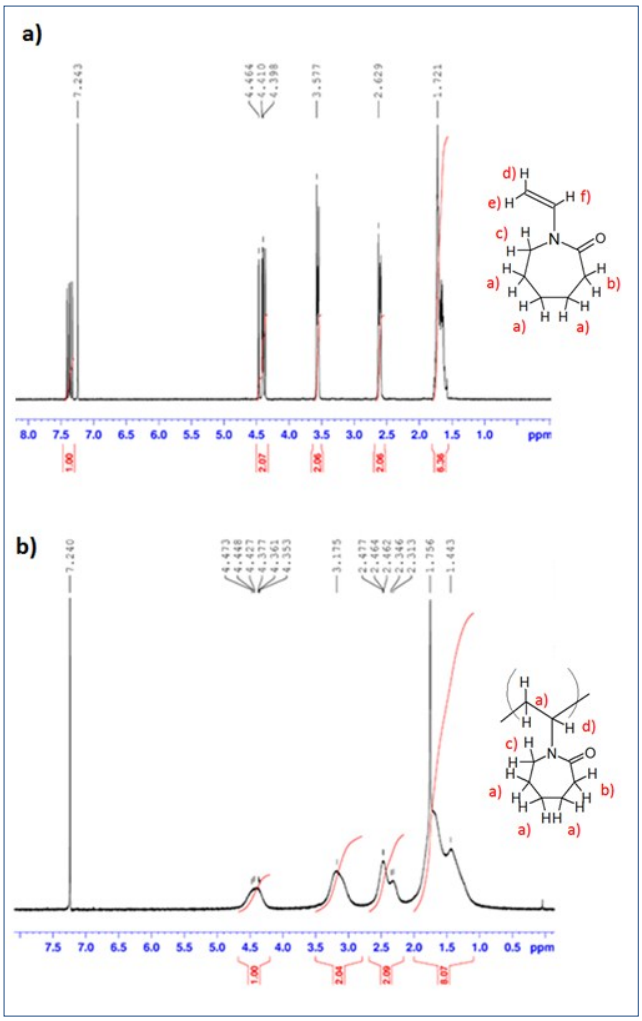

Figure S1. ^{1}H-NMR spectra of a) VCL and b) PVCL

Table S1. Peak assignment for ^{1}H-NMR spectra of VCL and

Proton type	Group	Shift (ppm)	
		VCL	PVCL
H_a	methylene	1.721	1.443, 1.756
H_b	methylene	2.629	2.313 – 2.477
H_c	methylene	3.577	3.175
H_d	ethylene	4.398	4.353 – 4.473
H_e	ethylene	4.464	-
H_f	ethylene	7.243	-

2. Viscosity average molecular weight determination

The viscosity average molecular weight (M_v) of the synthesised PVCL was determined from the intrinsic viscosity ([η]) by employing the Mark-Houwink relation $[\eta] = K*M_v^a$, with K = 0.0105 ml/g and a = 0.69.[3] An Ubbelohde-type viscometer was used at 25˚C with water as the solvent. The reduced viscosity (η_{red}) and inherent viscosity (η_{inh}) were determined and the

average of the Y-intercepts was used to calculate the [η] (Figure S1). Using the Mark-Houwink relation, the M_v was thus determined to be 35.4 kDa.

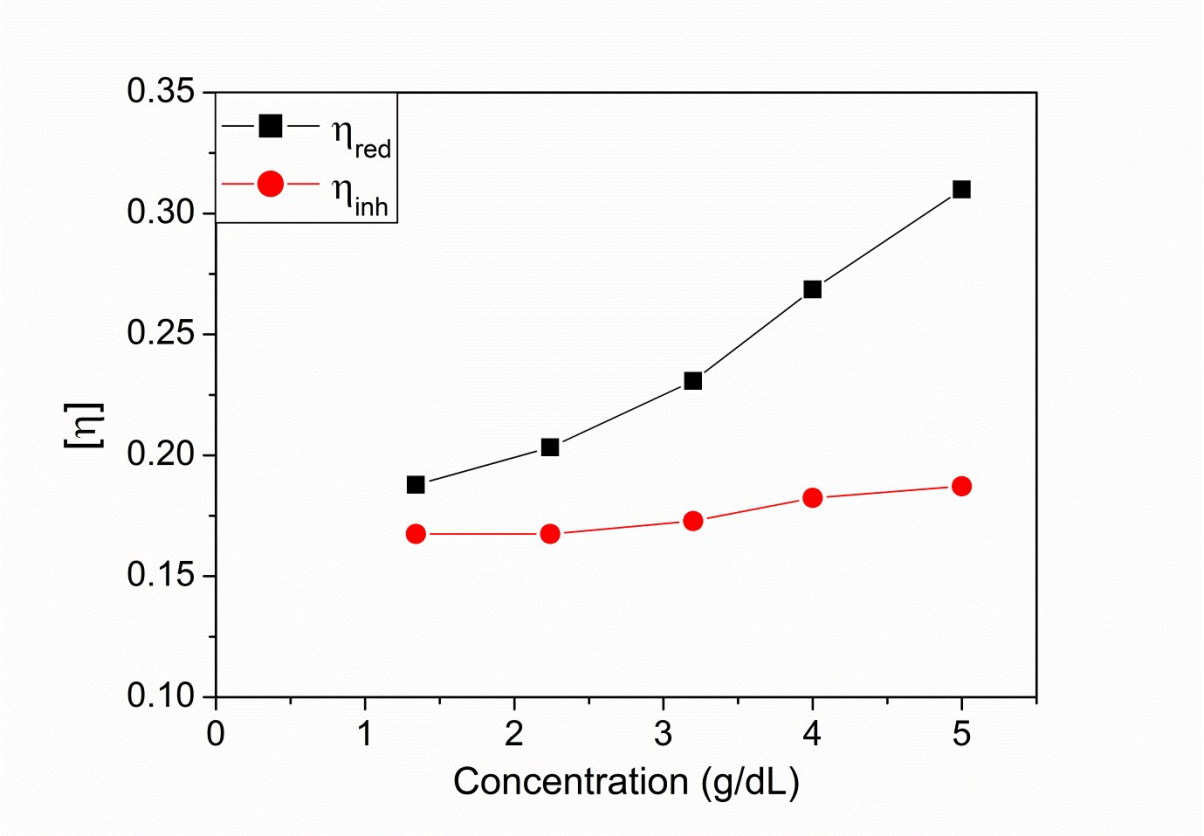

Figure S2. Plot of reduced and inherent viscosities determined for PVCL.

3. Calculation of mass ratio of RSF and PVCL in hydrogels by TGA

The TGA thermograms and corresponding derivative curves for the RSF hydrogel, PVCL and RSF/PVCL DN hydrogels at different rations are shown in Figure S3 below. The proportions of RSF and PVCL were estimated by taking the mass % at the plateau of each derivative. The residual mass was attributed to RSF. The calculations below represent the method of estimating the proportion of RSF and PVCL in the DN hydrogels.

.

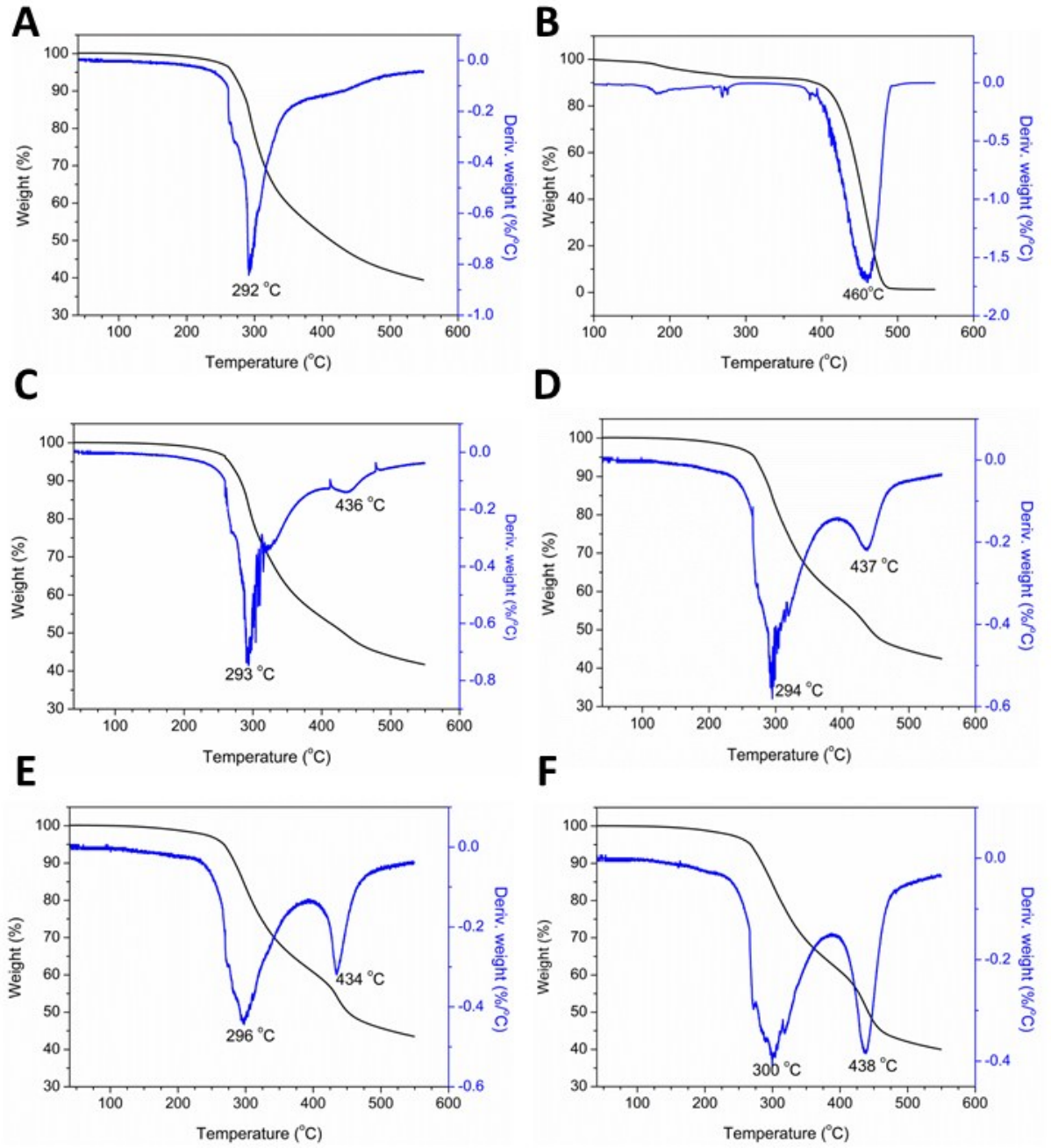

Figure S3. TGA thermograms of RSF/PVCL hydrogels prepared with different compositions. A) RSF; B) PVCL; C) $S_{90}P_{10}$; D) $S_{80}P_{20}$; E) $S_{70}P_{30}$; F) $S_{60}P_{40}$.

Calculations

PVCL residual from TGA thermogram: 1.24%

RSF residual from TGA thermogram: 39.4%

S90P10 TGA thermogram

Residual: 41.7

RSF: 99.38-53.3=46+41.7=87.7

PVCL: 53.3-41.7=11.6

RSF:PVCL = 7.6:1

S80P20 TGA thermogram

Residual: 42.5

RSF: 98.7-59.1=39.6+42.5=82.1

PVCL: 59.1-42.5=16.6

RSF:PVCL = 4.9:1

S70P30 TGA thermogram

Residual: 43.5

RSF: 98.7-61.8=36.9+43.5=80.4

PVCL: 61.8-43.5=18.3

RSF:PVCL = 4.4:1

S60P40 TGA thermogram

Residual: 40

RSF: 98.8-63.1=35.7+40=75.7

PVCL: 63.1-40=23.1

RSF:PVCL = 3.3:1

4. PCR primers

Table S2 Oligonucleotide sequences used for Real-time PCR

Gene	**Forward (5' – 3')**	**Reverse (5' – 3')**	**Mw (Da)**
β-actin	TTGCTGACACCATGCAGAAG	AAGGGTGTAAAACGCAGCTC	6126.1 /6175.1
Col I	AAAGGGTCCCTCTGGAGAAC	CACCTCTAGAGCCAGGGAGAC	6151.0 /6425.2
Col II	CGAGTGGAAGAGCGGAGACTAC	CCAGTTTTTCCGAGGGACAGT	6858.5 /6437.2
Sox9	ACGGCTCCAGCAAGAACAAG	TTGTGCAGATGCGGGTACTG	6129.0 /6204.1
Runx2	ATGATGACACTGCCACCTCTG	ATGAAATGCTTGGGAACTGC	6366.2 /6181.1
Sparc	ACTACATCGGACCATGCAAATAC	CGTACAAGGTGACCAGGACAT	6985.6 /6464.3
Bglap	AAGCAGGAGGGCAATAAGGT	TCAAGCCATACTGGTCTGATAGC	6264.2 /7023.7
Sp7	ATGGCGTCCTCTCTGCTTG	GTCCATTGGTGCTTGAGAAGG	5761.8 /6517.3
Ibsp	CAGTCCAGGGAGGCAGTG	GGAAAGTGTGGAGTTCTCTG	5589.7 /6228.1
Spp1	AGCAAGAAACTCTTCCAAGCAA	GATTCGTCAGATTCATCCGAGT	6705.4 /6725.4
Alpl	GCCTTACCAACTCTTTTGTGC	GGCTACATTGGTGTTGAGCTT	6323.2 /6483.3

5. Hydrogel degradation in PBS

The *in vitro* degradation profile of the RSF and RSF/PVCL hydrogels was examined in PBS over a period of 21 weeks and the results of the mass change over time is shown in Figure S4. There is negligible mass change for all samples over this period. Horan et al. observed that there was no significant weight loss of RSF samples incubated in PBS over a period of 42 days.[4] However, She et al. observed that there was a 19.28% weight loss for a RSF/chitosan scaffold incubated in PBS at 37˚C for 8 weeks.[5] They attributed the weight loss to the degradation of chitosan as the degradation rate of chitosan is much faster than RSF and the chitosan content was observed to decrease during the degradation process.[5] Thus, as the RSF hydrogels and RSF/PVCL DN hydrogels exhibited negligible weight loss over the 21 week period it can be concluded that the covalent di-tyrosine crosslinks and strong hydrogen bonding formed a robust structure that is stable over an extended period of time.

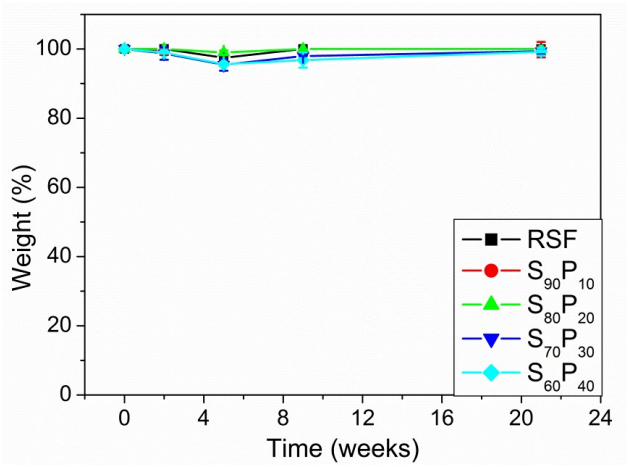

Figure S4. Mass of RSF and RSF/PVCL hydrogels in PBS as a function of time. Data is presented as mean ± standard deviation.

6. Gene analysis for chondrogenic and osteogenic markers on RSF hydrogels and TCP

The potential of the RSF hydrogel for use in cartilage tissue engineering was evaluated by examining the chondrogenic differentiation of the ATDC5 cells when cultured for 1, 7 and 14 days on the RSF samples and TCP surfaces. The ATDC5 cells were seeded at a density of 8.3 x 10^3 cells/cm^2 and supplemented with media containing the cartilage-inducing growth factor TGF-β1 (10 ng/mL).[6-8] TGF-β1 has been reported to accelerate chondrogenic differentiation at the early stages of culture.[9] The RNA was extracted from the cells at 1, 7 and 14 days, followed by cDNA synthesis. The expression of cartilage-related genes (Col I, Col II and Sox9) was quantified by performing real-time PCR. For all samples, β-actin was employed as the housekeeping gene. Figure S5 summarises the relative expression levels of the cartilage-related genes at 1, 7 and 14 days of culture on the RSF hydrogel and TCP surfaces. The expression levels of Col I, Col II and Sox9 in ATDC5 cells cultured on RSF were comparable to the TCP surfaces. Two-way ANOVA analysis with Sidak's multiple comparison test confirmed that there was no statistical difference between the expression of Col I, Col II and Sox9 on RSF and TCP at days 1, 7 and 14. These findings support the fact that the RSF hydrogels could potentially be used in cartilage tissue engineering applications. However, in order to improve the differentiation of chondrocytes in the presence of RSF hydrogels, it may be necessary to tune the stiffness and surface characteristics of the hydrogel as these have been shown to be integral for chondrogenic differentiation.[10] In addition, increasing the pore size and level of porosity of the hydrogels has also been shown to significantly improve the expression of Col II in RSF-based materials.[7, 11] However, the level of porosity has been

shown to be related to the water uptake behaviour and mechanical strength of the hydrogel and thus the right balance of properties should be maintained.

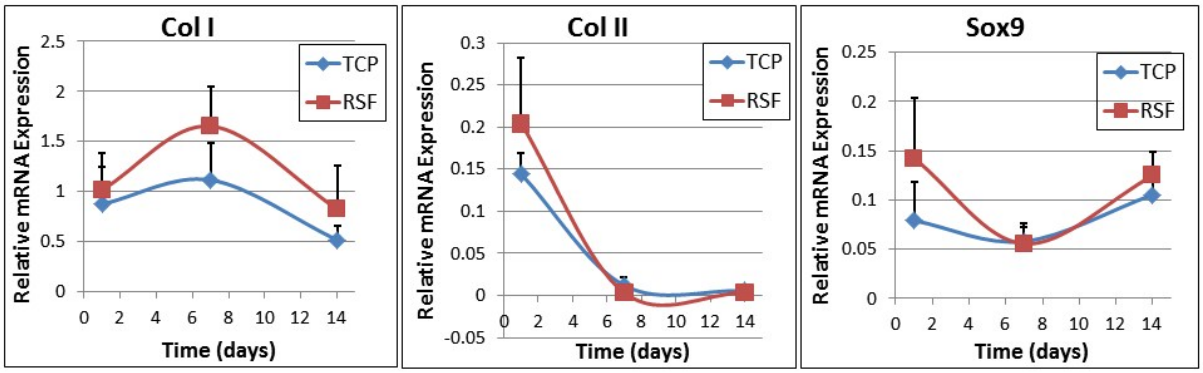

Figure S5. Real-time PCR analysis of cartilage-related gene expression by ATDC5 cells on RSF and control TCP after 1, 7 and 14 days of culture with induction media. Data is presented as mean + standard deviation.

In order to confirm that the RSF hydrogels could be used for cartilage tissue engineering applications, the osteogenic potential of the ATDC5 cells when cultured on the hydrogels was evaluated using RT-PCR to ensure there was no tendency for osteogenic differentiation. As with the chondrogenic study, the ATDC5 cells were cultured with inductive media and the RNA was extracted at days 1, 7 and 14, followed by cDNA synthesis and RT-PCR. The osteogenic markers investigated included Runx2, osteonectin (Sparc), osteocalcin (Bglap), osterix (Sp7), integrin-binding saloprotein (Ibsp), osteopontin (Spp1), and alkaline phosphatase (Alpl). Figure S6 depicts the relative expression of the osteogenic markers for the ATDC5 cells grown on the RSF hydrogels and TCP at 1, 7 and 14 days. It was observed that there were no statistical differences in the expression of Runx2, Sp7, Ibsp and Spp1 for the cells cultured on RSF and TCP as confirmed by two-way ANOVA analysis with Sidak's multiple comparisons test. The cells cultured on TCP exhibited significantly higher expression of Sparc at day 1 (~2.5) relative to β-actin, compared to RSF (~0.9). Also, the expression of Bglap after 14 days of culture was significantly higher on TCP (~0.005) relative to β-actin, compared to RSF (~0.002). In addition, expression of Alpl was statistically higher for the cells cultured for 1 and 7 days on RSF compared to TCP. These results further support the proposed use of the RSF hydrogels for cartilage tissue regeneration applications. As discussed above, there is further scope to tune the hydrogel properties in order to stimulate a more favourable cellular response for cartilage regeneration applications.

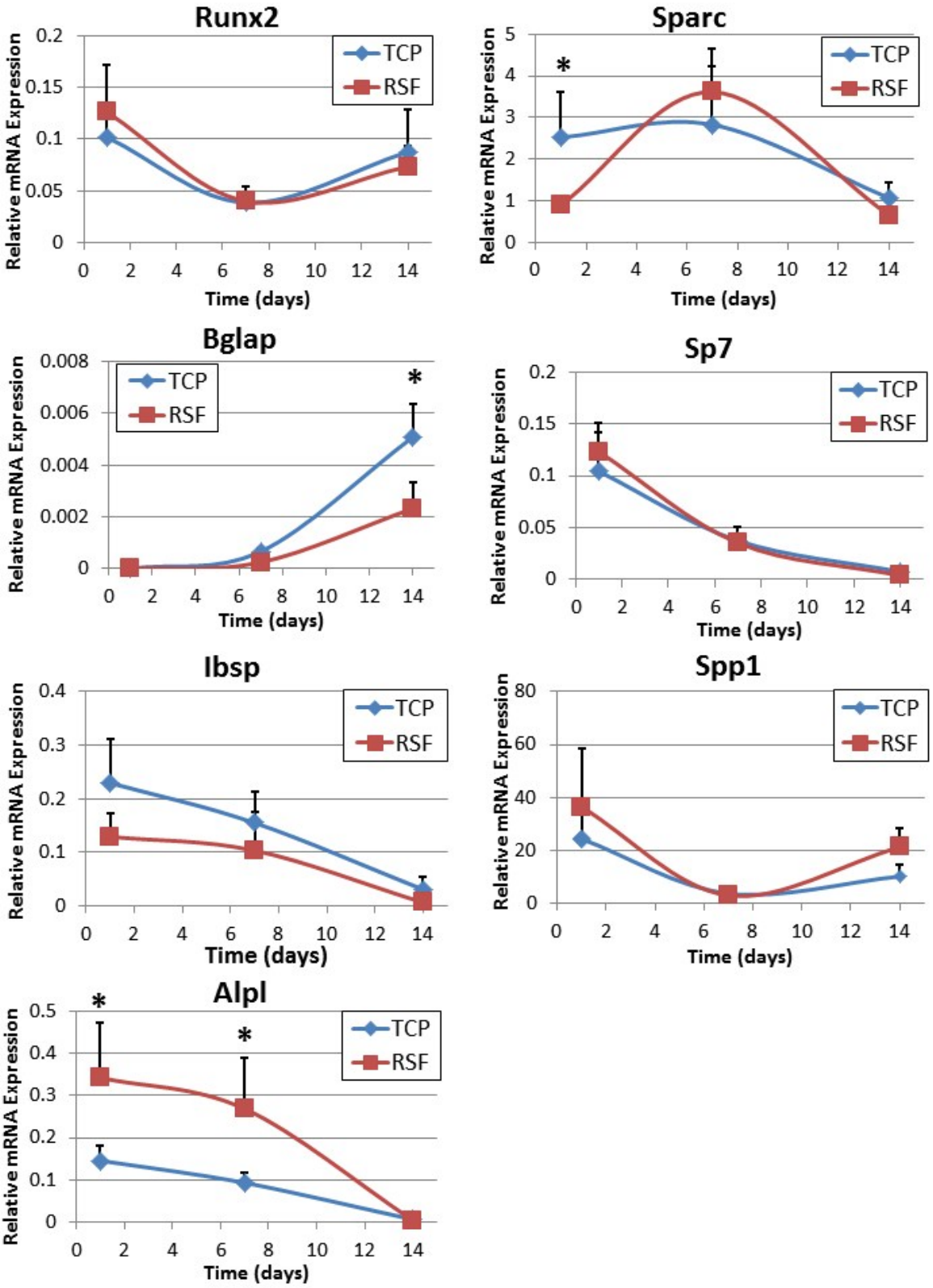

Figure S6. Real-time PCR analysis of osteogenic-related gene expression by ATDC5 cells on RSF and control TCP after 1, 7 and 14 days of culture with induction media. * Represents groups that are statistically different ($p<0.05$).

References

1. Webster, M.; Miao, J.; Lynch, B.; Green, D. S.; Jones-Sawyer, R.; Linhardt, R. J.; Mendenhall, J., Macromolecular Materials and Engineering **2013**, 298 (4), 447-453.

2. Kozanoglu, S.; Ozdemir, T.; Usanmaz, A., Journal of Macromolecular Science Part A: Pure and Applied Chemistry **2011,** 48 (6), 467-477.

3. Loos, W.; Verbrugghe, S.; Goethals, E. J.; Du Prez, F. E.; Bakeeva, I. V.; Zubov, V. P., Macromolecular Chemistry and Physics **2003,** 204 (1), 98-103.

4. R. L. Horan, K. Antle, A. L. Collette, Y. Wang, J. Huang, J. E. Moreau, V. Volloch, D. L. Kaplan, G. H. Altman, *Biomaterials*, 2005, **26** (17), 3385-3393.

5. Z. She, B. Zhang, C. Jin, Q. Feng, Y. Xu, *Polym. Degrad. Stab.*, 2008, **93** (7), 1316-1322.

6. Y. Wang, D. J. Blasioli, H.-J. Kim, H. S. Kim, D. L. Kaplan, *Biomaterials*, 2006, **27** (25), 4434-4442.

7. R. Seda Tığlı, S. Ghosh, M. M. Laha, N. K. Shevde, L. Daheron, J. Gimble, M. Gümüşderelioğlu, D. L. Kaplan, *J. Tissue Eng. Regener. Med.*, 2009, **3** (5), 348-360.

8. F. Barry, R. E. Boynton, B. Liu, J. M. Murphy, *Exp. Cell Res.*, 2001, **268** (2), 189-200.

9. F. Han, C. S. Adams, Z. Tao, C. J. Williams, R. Zaka, R. S. Tuan, P. A. Norton, N. J. Hickok, *J. Cell. Biochem.*, 2005, **95** (4), 750-762.

10. H. Kwon, L. Sun, D. M. Cairns, R. S. Rainbow, R. C. Preda, D. L. Kaplan, L. Zeng, *Acta Biomater.*, 2013, **9** (5), 6563-6575.

11. Y. Wang, U.-J. Kim, D. J. Blasioli, H.-J. Kim, D. L. Kaplan, *Biomaterials*, 2005, **26** (34), 7082-7094.