Biological Chemistry & Chemical Biology

Thymosin-β4, and Human Vitronectin peptides Grafted to Collagen Tune Adhesion or VEGF Gene Expression in Human Cell Lines

Roberto Guizzardi,[a, b] Annj Zamuner,[c] Paola Brun,[d] Monica Dettin,[c] Antonino Natalello,[a] and Laura Cipolla*[a]

In order to improve collagen bioactivity for regenerative medicine approaches, thymosin-β4 (Tβ4P) and Human Vitronectin (HVP) derived peptides are grafted to collagen by thiol-ene Michael addition. Tβ4P and HVP are known to exert a pro-angiogenic and a pro-adhesive activity respectively and HVP is involved in osteogenesis promotion. The ability of these peptides to increase collagen cell adhesion and angiogenesis properties is assessed on human cell lines. In particular, HVP-grafted collagen increased human osteoblast adhesion and cell proliferation: after 24 h, both adhesion and proliferation roughly showed a 4-fold increase, if compared to pristine collagen. Tβ4P-grafted collagen promotes Vascular Endothelial Growth Factor (VEGF) gene expression in human vascular cell lines by more than 7 times. These results suggest that HVP-grafted collagen may be an interesting biomaterial for bone tissue regeneration, while Tβ4P-grafted collagen is useful for angiogenesis promotion.

Introduction

Collagen, the most copious protein found in mammals constituting the main structural protein of the extracellular matrix (ECM),[1] is finding wide application in several regenerative medicine strategies[2–4] either used individually or in combination with other materials of natural or synthetic origin.[5] The use of collagen-based biomaterials in tissue engineering approaches has been gaining significant interest over the last two decades due to its biocompatibility, biodegradability, availability, and versatility in scaffolds manufacturing (hydrogels, fibers, films).[6,7] In general, to promote tissue regeneration, an instructive microenvironment able to drive cell behavior and scaffold biodegradability are key issues: while the bioactive microenvironment stimulates cell proliferation and differentiation towards tissue regeneration, the biomaterial used as the scaffold slowly degrades leaving room to the newly formed tissue. A possible approach to material bioactivation is the integration of growth factors,[8,9] short signaling peptides,[10–12] or carbohydrate signalling cues.[13–15]

More specifically, cell-material interactions promoting adhesion are fundamental for scaffold colonization; in addition regeneration relies on angiogenesis and tissue vascolarization that is essential for the delivery of oxygen and nutrients throughout the engineered constructs. Collagen shows intrinsic adhesive properties due to the presence of several adhesive sequences in its primary structure; however, the modulation of adhesiveness strength and specificity of collagen scaffolds thorugh the grafting of adhesive peptides may improve both cell adhesion and migration, favouring the tissue regenerative process.[16] On the other hand, collagen on its own lacks pro-angiogenic properties.[17] Thus, the covalent grafting of angiogenic and adhesive bioactive molecules to collagen matrices was envisaged as an interesting strategy to improve collagen bioactivity for tissue regeneration approaches.

Thymosin-β4 peptide (Tβ4P)[18] and Human Vitronectin Peptide (HVP),[19,20] are suitable peptides towards this aim. In fact, Tβ4P[18] and HVP[19,20] exert respectively a pro-angiogenic and a pro-adhesive activity through interaction with specific actin binding sites, promoting Vascular Endothelial Growth Factor (VEGF) and filopodia expression resulting in attachment and spreading of endothelial cells or osteoblasts. In particular, VEGF induces cell proliferation, cell migration, and inhibits apoptosis.[21] The effect of HVP in *h*-osteoblast survival and proliferation has been extensively investigated.[19,22, 23] As reported, HVP peptide promotes cell adhesion during the first

[a] *R. Guizzardi, A. Natalello, L. Cipolla*
Dept. of Biotechnology and Biosciences,
University of Milano-Bicocca, P.zza della Scienza 2,
20126 Milano-Italy
E-mail: laura.cipolla@unimib.it
[b] *R. Guizzardi*
Present address: Tecnoservizi ambientali s.r.l.
[c] *A. Zamuner, M. Dettin*
Dept. of Industrial Engineering,
University of Padova, Via Marzolo, 9,
35131 Padova-Italy
[d] *P. Brun*
Dept. of Molecular Medicine,
University of Padova,
Via Gabelli, 63, 35121 Padova-Italy

[**] *VEGF, Vascular Endothelial Growth Factor*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/slct.202102757

step of cell seeding and selects osteoblasts for attachment through proteoglycan-mediated interactions.

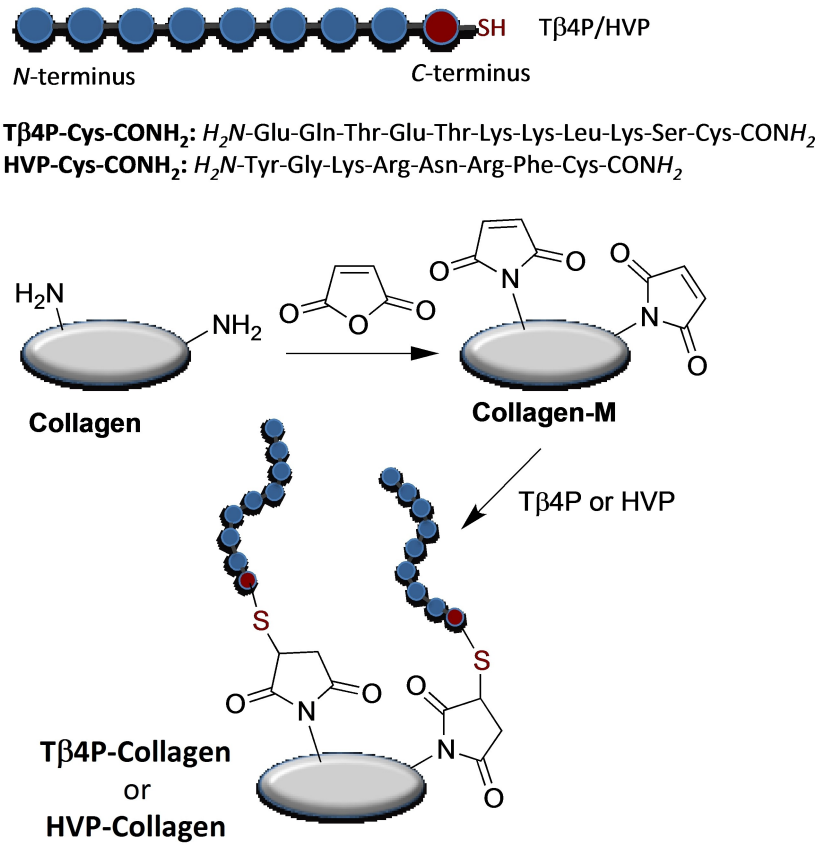
Several examples of collagen bioactivation do exists with several biomolecules,[24] such as signalling carbohydrates,[25,26] growth factors,[27] and short bioactive peptides,[28–31] however this work proposes for the first time collagen covalent functionalization with the angiogenic peptide Tβ4P and the pro-adhesive peptide HVP.

Results and discussion

In order to covalently graft the peptide sequences to collagen coatings, thiol-Michael addition click chemistry[32] was considered a suitable tool to reach the goal. (Scheme 1).

The thiol-maleimide reaction is particularly attractive due to its efficiency in aqueous environments, its rapid kinetic, and the stability of the thiol-maleimide conjugation product. Thus, the selected peptides were synthesised with a C-terminal cysteine residue in order to introduce the donor functionality, while collagen was derivatized with a maleimido group taking advantage of lysine side chains. Solid Phase Peptide Synthesis (SPPS) afforded the peptides Tβ4P-Cys and HVP-Cys (64% and 72% respectively) which were opportunely purified by reverse-phase chromatography before conjugation. On the other hand, collagen lysine amino groups were converted into maleimido groups, as the Michael acceptors, reacting collagen coatings with maleic anhydride (Collagen-M, Scheme 1). The peptides were finally coupled by Michael addition to collagen-M coatings affording Tβ4P-Collagen and HVP-Collagen.

The effectiveness of the collagen functionalization by maleic anhydride and the subsequent Michael addition was assessed by FTIR. The ATR-FTIR absorption spectra of the different collagen coating samples display the typical spectral features of polypeptides and are characterized by the Amide I and Amide II bands (Figure 1). A new absorption peak around 868 cm^{-1} appears after the maleimido functionalization of pristine collagen (Figure 1 A), as can be better appreciated in the second derivative spectra (Figure 1B and 1D), whose minima correspond to absorption maxima. This peak can be assigned to the =C–H bending vibration of the maleimide group.[33] The Michael addition reactions for the two peptides were therefore followed by the depletion of the substrate's absorption peak around 868 cm^{-1}.[33] The decrease of this band in Tβ4P-Collagen and HVP-Collagen samples (Figure 1B and 1D) can be ascribed to the addition reaction between the peptide –SH and maleimide –C=C– group in the thiol-Michael reactions (Figure 1B and 1D). In comparison with the pristine collagen, the HVP-Collagen sample displays an intensity increase around 1514 cm^{-1} (Figure 1 C, 1E), in the typical spectral region of tyrosine absorption.[34] Since collagen lacks tyrosines (less than 0.75%)[35] and since HVP contains one tyrosine out of 8 amino



Scheme 1. Peptides grafting strategy to collagen coatings.

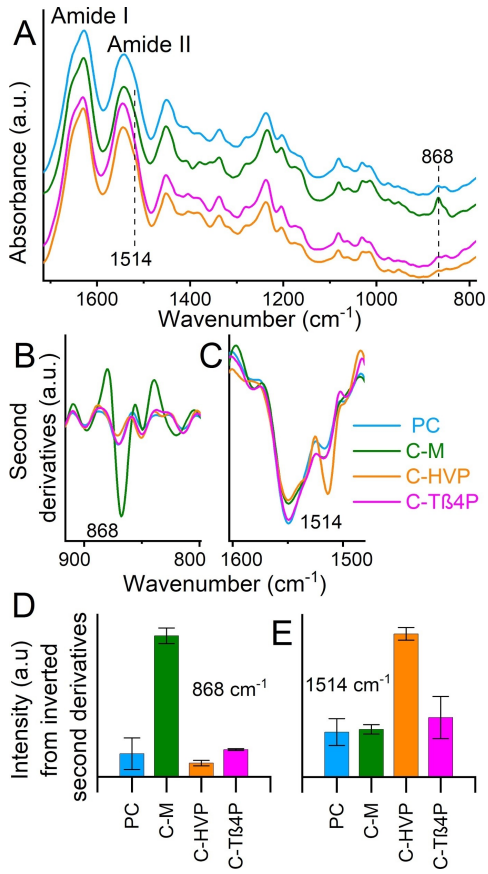


Figure 1. ATR-FTIR characterization of collagen coating samples. A) ATR-FTIR absorption spectra. B, C) Second derivatives of the ATR-FTIR absorption spectra reported in the 900–800 cm^{-1} and 1600–1500 cm^{-1} region. D, E) Intensities of the ~868 cm^{-1} and ~1514 cm^{-1} peaks taken from the inverted second derivative spectra. PC, Pristine Collagen; C–M, Collagen-Maleimido; C-HVP, HVP-Collagen; C–Tβ4P, Tβ4P-Collagen.

acids, this spectral change further confirms the HVP conjugation, which indeed involves tyrosine insertion.

Collagen grafted with Tβ4P-Cys and HVP-Cys (namely Tβ4P-Collagen and HVP-Collagen) were assessed for their ability to promote angiogenesis and cell adhesion respectively, if compared to pristine collagen.

The improved adhesiveness of HVP-functionalized collagen was assayed using primary human (*h*) osteoblast cells from cortical mandible bone. The adhesion of *h*-osteoblasts was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay that interrogates the cell metabolic activity. Indeed, the tetrazolium dye MTT (yellow) is reduced by the NAD(P)H-dependent cellular oxidoreductase enzymes to generate the insoluble formazan salt (purple color) which presence is directly proportional to the number of viable cells adhering to the matrices. As reported in Figure 2A, adhesion of *h*-osteoblast was significantly greater in HVP-Collagen than pristine collagen. Indeed, the increased cell adhesion was evident after 2 h in culture and peaked 24 h later.

Besides cell adhesion, HVP-Collagen supports cell proliferation that was investigated by loading the cells with the carboxyfluorescein succinimidyl ester (CFSE). CFSE is a fluorescent, cell permeable dye that covalently couples to intracellular lysine residues and other amine sources via its succinimidyl group. Due to this covalent coupling reaction, CFSE is retained within cells for long periods and partitioned equally among daughter cells. As shown in Figure 2B, following 24 h in culture CFSE-related fluorescence was reported in 86.71 ± 1.5 % of *h*-osteoblasts cultured on HVP-Collagen, whereas only 22.09 ± 1.2 % of CFSE-positive cells in pristine collagen culture was detected.

To investigate the pro-angiogenic activity, Tβ4P-Collagen was seeded with Human Dermal Lymphatic Endothelial Cells (HDLEC) purchased from ScienCell and cultured in Endothelial Cell Medium. Following 48 h in culture, specific mRNA transcript levels coding Vascular Endothelial Growth Factor (*VEGF*) were quantified. As reported in Figure 3A, specific mRNA *VEGF* levels increased in cells cultured on pristine collagen (data are reported as folds over cells cultured on plastic support), whereas Tβ4P-Collagen increased expression of *VEGF* gene by more than 7 times.

Tβ4P-Collagen was not endowed with proliferative capabilities towards HDLEC cells (Figure 3B), whereas HVP-Collagen did not increase *VEGF* mRNA transcript levels in *h*-osteoblasts (data not shown), leading to the hypothesis that different collagen functionalization is able to drive specific bioactivities.

These results demonstrate that it is possible to improve collagen-based matrices with specific peptides able to select the cells more suitable for biomaterial colonization, thus speeding up the integration process of collagen-coated implants. As the functionalization process described in the present work is easily scalable, the combined use of HVP and Tβ4P peptides for the collagen matrices functionalization can be exploited to promote simultaneously the pro-angiogenic and the osteogenic activities that are mandatory during the bone healing process.

Conclusions

Despite the chemical nature of collagen, being a polypeptide itself, its conjugation to short bioactive peptide such as Thymosin β-4 peptide (Tβ4P) or Human Vitronectin Peptide (HVP) resulted in a clear improvement of collagen bioactivity, behaving as a matrix able to drive cell response, useful for regenerative medicine applications. In particular, HVP-Collagen showed increased human osteoblast cells adhesion than pristine collagen, while Tβ4P-Collagen promoted the expression of *VEGF* gene in human vascular cell lines. This observation is particularly interesting in light of bone tissue engineering since vascularization is mandatory in bone regeneration. Results suggest further studies on collagen bioactivation by grafting both peptides and evaluating if adhesion, proliferation and angiogenesis gene expression are promoted at the same time. In addition, more studies about peptide concentrations, their topological localization on the matrice surfaces, enzymatic

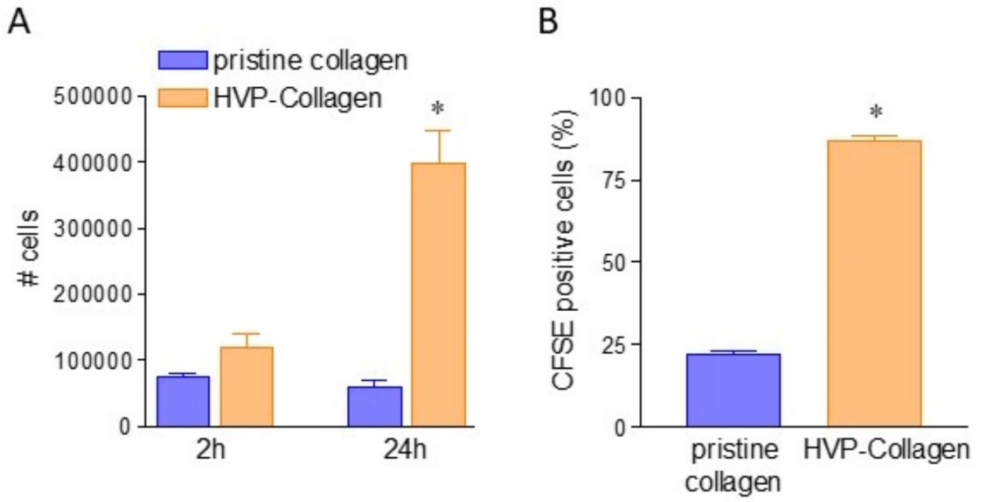


Figure 2. HVP-Collagen drives adhesion and proliferation of *h*-osteoblasts. Adhesion was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test following 2 or 24 h of culture. Cell proliferation was evaluated by flow cytometry analysis in CFSE (carboxyfluorescein succinimidyl ester) loaded cells. * denotes $P < 0.02$ *vs* pristine collagen at the same culture time. Data are reported as mean ± standard error of two independent experiments, each performed in triplicate. Statistical analysis was performed using the ONE-way ANOVA test followed by Bonferroni's multicomparison test.

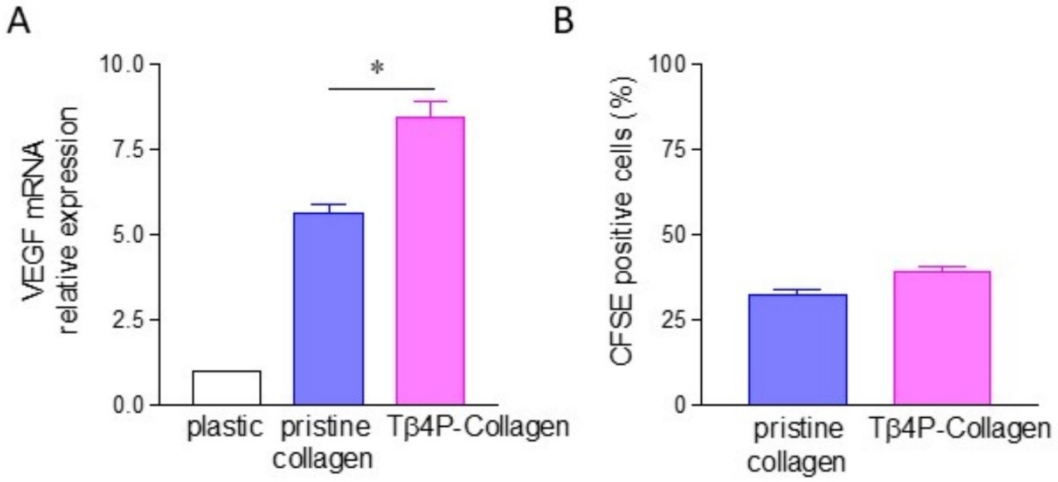


Figure 3. Tβ4P-Collagen induces expression of *VEGF* in Human Dermal Lymphatic Endothelial Cells (HDLEC). Specific *VEGF* mRNA transcript levels were assessed in HDLEC cultured for 48 h. Data were calculated as fold expression over cells cultured on plastic supports. Proliferation of HDLEC cultured for 24 h on Tβ4P-Collagen was evaluated by flow cytometry analysis in CFSE loaded cells. *denotes $P < 0.02$ *vs* pristine collagen at the same culture time. Data are reported as mean ± standard error of two independent experiments, each performed in duplicate. Statistical analysis was performed using the ONE-way ANOVA test followed by Bonferroni's multicomparison test.

degradation by cellular enzymes, need to be taken into account in future studies.

Supporting Information Summary

Detailed Experimental Section including detailed protocols, physico-chemical and biological characterization.

Acknowledgements

The authors gratefully acknowledge fundings by Ministero dell'Istruzione e della Ricerca, (MIUR), under project PRIN 2010L9SH3K Open Access Funding provided by Universita degli Studi di Milano-Bicocca within the CRUI-CARE Agreement.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: collagen · covalent grafting · growth factor · human osteoblasts · human dermal lymphatic endothelial cells · human vitronectin · peptides · thymosin-β4

[1] M. D. Shoulders, R. T. Raines, *Annu. Rev. Biochem.* **2009**, *78*, 929–958.
[2] E. A. Abou Neel, L. Bozec, J. C. Knowles, O. Syed, V. Mudera, R. Day, J. K. Hyun, *Adv. Drug Delivery Rev.* **2013**, *65*, 429–456.
[3] R. Parenteau-Bareil, R. Gauvin, F. Berthod, *Materials* **2010**, *3*, 1863–1887.
[4] D. Miranda-Nieves, E. L. Chaikof, *ACS Biomater. Sci. Eng.* **2017**, *3*, 694–711.
[5] C. Dong, Y. Lv *Polymer* **2016**, *8*, 42.
[6] G. E. Rezvani, N. Nourbakhsh, K. N. Akbari Kenari, M. Zare, S. Ramakrishna, *J. Biomed. Mater. Res. Part A* **2021**, 1–14.
[7] K. M. Pawelec, S. M. Best, R. E. Cameron, *J. Mater. Chem. B* **2016**, *4*, 6484–6496.
[8] S. Ohba, H. Hojo, U. Chung, *Muscles Ligaments Tendons J.* **2012**, *2*, 193–203.
[9] K. Lee, E. A. Silva, D. J. Mooney, *J. R. Soc. Interface* **2011**, *8*, 153–170.
[10] C. Wang, Y. Liu, Y. Fan, X. Li, *Regener. Biomat.* **2017**, 191–206.
[11] J. Y. Lee, Y. S. Choi, S. J. Lee, C. P. Chung, Y. J. Park, *Curr. Pharm. Des.* **2011**, *17*, 2663–2676.
[12] K. G. Sreejalekshmi, P. D. Nair *J. Biomed. Mater. Res. Part A* **2011**, *96*, 477–491.
[13] L. Russo, L. Cipolla *Chem. Eur. J.* **2016**, *22*, 13380–13388.
[14] S. A. Kalovidouris, C. I. Gama, L. W. Lee, L. C. Hsieh-Wilson, *J. Am. Chem. Soc.* **2005**, *127*, 1340–1341.
[15] V. Secchi, R. Guizzardi, L. Russo, V. Pastori, M. Lecchi, S. Franchi, G. Iucci, C. Battocchio, L. Cipolla, *J. Mol. Struct.* **2018**, *1159*, 74–78.
[16] G. A. Monteiro, A. V. Fernandes, H. G. Sundararaghavan, D. I. Shreiber, *Tissue Eng. Part A* **2011**, *17*, 1663–1673.
[17] J. Sottile, *Biochim. Biophys. Acta Rev. Cancer* **2004**, *1654*, 13–22.
[18] M. Dettin, F. Ghezzo, M. T. Conconi, L. Urbani, G. D'Auria, L. Falcigno, D. Guidolin, B. Nico, D. Ribatti, C. Di Bello, *P. P. Cell. Immunol.* **2011**, *271*, 299–307.
[19] P. Brun, M. Scorzeto, S. Vassanelli, I. Castagliuolo, G. Palù, F. Ghezzo, G. M. Messina, G. Iucci, V. Battaglia, S. Sivolella, A. Bagno, G. Polzonetti, G. Marletta, M. Dettin, *Acta Biomater.* **2013**, *9*, 6105–6115.
[20] D. Philp, T. Huff, Y. S. Gho, E. Hannappel, H. K. Kleinman, *FASEB J.* **2003**, *17*, 2103–2105.
[21] G. Neufeld, T. Cohen, S. Gengrinovitch, Z. Poltorak, *FASEB J.* **1999**, *13*, 9–22.
[22] P. Brun, A. Zamuner, C. Battocchio, L. Cassari, M. Todesco, V. Graziani, G. Iucci, M. Marsotto, L. Tortora, V. Secchi, M. Dettin, *Int. J. Mol. Sci.* **2021**, *22*, 5916.
[23] A. Zamuner, P. Brun, M. Scorzeto, G. Sica, I. Castagliuolo, M. Dettin, *Bioact. Mater.* **2017**, *2*, 121–130.
[24] L. Cipolla, in *Soft Matter for Biomedical Applications*, Soft Matter Series No. 13 (Eds: H.S Azevedo, J. F. Mano, J. Borges), Royal Society of Chemistry **2021**, 61.
[25] L. Russo, A. Sgambato, M. Lecchi, V. Pastori, M. Raspanti, A. Natalello, S. M. Doglia, F. Nicotra, L. Cipolla, *ACS Chem. Neurosci.* **2014**, *5*, 261–265.
[26] A. Sgambato, L. Russo, M. Montesi, S. Panseri, M. Marcacci, E. Caravà, M. Raspanti, L. Cipolla, *ACS Appl. Mater. Interfaces* **2016**, *8*, 14952–14957.
[27] H. J. Lee, G. M. Fernandes-Cunha, I. Putra, W.-G. Koh, D. Myung *ACS Appl. Mater. Interfaces* **2017**, *9*, 23389–23399.
[28] Y. Xiao, L. A. Reis, Y. Zhao, M. Radisic, *Methods* **2015**, *84*, 44–52.
[29] G. A. Monteiro, A. V. Fernandes, H. G. Sundararaghavan, D. I. Shreiber, *Tissue Eng. Part A* **2011**, *17*, 1663–1673.
[30] K. C. Olbrich, T. T. Andersen, F. A. Blumenstock, R. Bizios, *Biomaterials* **1996**, *17*, 759–764.
[31] J. L. Myles, B. T. Burgess, R. B. Dickinson, *J. Biomat. Sci., Polym. Ed.* **2000**, *11*, 69–86.
[32] D. P. Nair, M. Podgórski, S. Chatani, T. Gong, W. Xi, C. R. Fenoli, C. N. Bowman, *Chem. Mater.* **2014**, *26*, 724–744.
[33] L.-T. T. Nguyen, M. Talha Gokmenac, F. E. Du Prez *Polym. Chem.* **2013**, *4*, 5527–5536.
[34] A. Barth *Prog. Biophys. Mol. Biol.* **2000**, *74*, 141–173.
[35] D. J. Hulmes, A. Miller, D. A. Parry, K. A. Piez, J. Woodhead-Galloway, *J. Mol. Biol.* **1973**, *79*, 137–148.

Submitted: August 5, 2021
Accepted: September 24, 2021