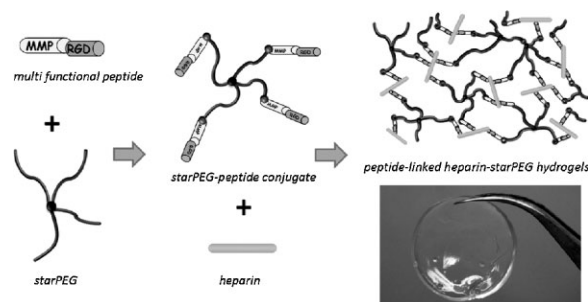


Modular StarPEG-Heparin Gels with Bifunctional Peptide Linkers^{a,b}

Mikhail V. Tsurkan, Karolina Chwalek, Kandice R. Levental, Uwe Freudenberg, Carsten Werner*

Cell responsive materials are instrumental to regenerative therapies. Here, we report about a novel biohybrid hydrogel that consists of heparin and peptide-conjugated star-shaped poly(ethylene glycol), crosslinked by peptide units that combine matrix metalloproteinase (MMP) sensitivity and cell adhesive modules. Taking advantage of the high affinity of vascular endothelial growth factor to heparin, we illustrate the applicability of our hydrogels as a novel system that is supportive of cellular remodeling and three-dimensional migration of human endothelial cells.



Introduction

As cellular fate decisions are controlled by exogenous signals presented through extracellular matrices (ECMs), the design of bioartificial matrices receives particular attention in research toward regenerative therapies.^[1–3] Naturally occurring ECMs consist of complex supramolecular assemblies of proteins, proteoglycans, and glycosaminoglycans and provide mechanical support to modulate cell signaling and undergo dynamic changes through cell-mediated remodeling. Engineered matrices should mimic these characteristics closely in order to effectively support cellular growth and differentiation. To meet this criterion,

recent studies implemented multiple sets of ECM components,^[4–7] combined ECM motifs in self-assembling peptide-amphiphile nanofibers^[8] and employed various advanced materials for the sequential release of signaling molecules.^[9,10] Among these approaches, the incorporation of polysaccharidic ECM components into synthetic scaffolds is of increasing interest^[11–17] to capitalize on their ability to associate with various signaling molecules (e.g., growth factors).^[18] Beyond that, the cell responsive remodeling of engineered matrix materials is crucial for various regeneration processes.^[19,20]

To address this challenge, we have developed a modular system of biohybrid hydrogels based on heparin and synthetic star-shaped poly(ethylene glycol) (starPEG). Key to our approach is the use of heparin, a naturally occurring highly anionic polysaccharide which has a high affinity to many important signaling molecules (e.g., growth factors, cytokines, ECM molecules).^[18] By using enzymatically cleavable peptide linkers, the naturally functional heparin and the biocompatible and flexible PEG are crosslinked together into a covalent three-dimensional network.^[21] By varying the degree of crosslinking, the viscoelastic characteristics and swelling of the hydrogel material can be controlled.^[22] The peptide linker used in this system is designed to be sensitive to the presence of matrix

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metalloproteinases (MMPs), thus permitting the degradation and remodeling of the hydrogel material by cells secreting this enzyme.^[23]

In the present work, we further extend the design of this modular biohybrid material through the implementation of bifunctional peptide linker units. Along with an MMP-sensitive sequence, a short amino acid sequence encoded as RGD, one of the most important ligands of cellular adhesion receptors which is found in several ECM proteins,^[24] was included in the peptide design. The incorporation of the RGD sequence was previously reported to trigger the adhesion of cells to PEG-based materials^[19] which are otherwise non-adhesive due to their protein-repulsive characteristics.^[25] The combination of enzymatically cleavable and cell adhesive peptides within one bifunctional linker was hypothesized to provide gel materials capable of effectively supporting matrix remodeling and, thus, facilitating the stimulation of angiogenesis.

Results and Discussion

The synthesis of the biohybrid hydrogels is summarized in Figure 1. First, the terminal hydroxyl functions of commercially available starPEG molecules were modified with acrylate groups, which were then stoichiometrically coupled through Michael-type addition with a cysteine residue of a synthetic apopeptide of custom design. In the final step, carboxylic groups of heparin were activated with carbodiimide/sulfo-*N*-hydroxysuccinimide (sulfo-NHS) to create a hydrogel network by the formation of amide bonds with *N*-terminal amino groups of the starPEG-peptide conjugate.

The applied EDC/sulfo-NHS chemistry limits the options to incorporate peptide sequences in gel materials. However, RGD moieties are commonly attached to various materials by carbodiimide chemistry^[26,27] and were therefore

expected to remain biologically active during the gel formation. Furthermore, the MMP sensitive peptide sequence GPQG↓IWGQ was demonstrated to maintain its activity after the inclusion into hydrogel networks by EDC/sNHS chemistry.^[21]

The sequences RGD and GPQG↓IWGQ were included as two bioactive modules into one peptide NH₂–GGRGDGPQGIWGQGGCG–CONH₂ (MMP-RGD). The peptide was synthesized by standard Fmoc solid-phase techniques on an amide resin (for details see Supplementary Information). The purity of the peptide was confirmed by detection of a single peak in analytical high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). ESI-MS shows two signals $m/z = 779.15$ and $m/z = 1557.27$ which correspond to the mono- and double-charged molecular ions of a species with a molecular weight 1556.30 (calculated: 1556.69). The purity of the peptide was greater than 90%.

For starPEG conjugation, the apopeptide MMP-RGD was dissolved in phosphate buffer (pH = 7.5) and added to the buffered solution of acryloyl-functionalized four-armed poly(ethylene glycol) (starPEG-Acl). A small amount of tris(2-carboxyethyl)phosphine chloride (TCEP) was added to the reaction mixture in order to prevent oxidation of the cysteine residue. The reaction mixture was stirred overnight and purified by dialysis (nitrocellulose membrane with MWCO = 3500 Da) against water several times. The white fluffy remnants were collected after lyophilizing the dialyzed solution. The purity of the formed starPEG-MMP-RGD conjugate was characterized by reverse phase HPLC set at 210 and 278 nm (Figure 2A). Absorption at 278 nm is characteristic of the tryptophan residue, but found to be absent in the spectrum of starPEG-Acl. The apopeptide MMP-RGD eluted in HPLC at 22 min while the starPEG-MMP-RGD conjugate eluted at 45 min. Both of the samples showed similar absorption at 210 and 278 nm. The single peak in the HPLC clearly suggests a complete reaction

through which all four arms of the starPEG-Acl were bound to four MMP-RGD peptides.

The procedure for the formation of the cleavable starPEG-MMP-RGD heparin gels was similar to the previously reported formation of non-cleavable starPEG-heparin gels.^[22] A two-fold excess of EDC and stoichiometric amounts of NHS were simultaneously added to the solution of heparin in water. The reaction mixture was kept for 15 min at 4 °C, and then, a stoichiometric amount of starPEG-peptide solution in water was added in order to reach the desired 28:08 ratio between carboxylic groups of heparin and the terminating amino

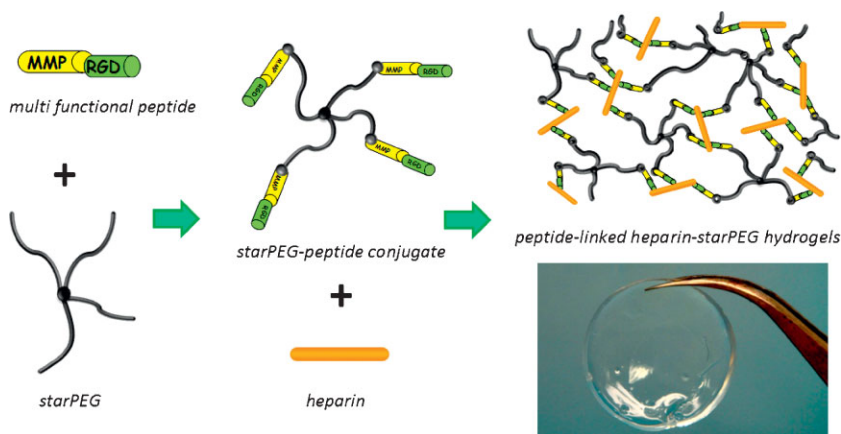


Figure 1. Schematic view of the formation of a starPEG-heparin hydrogels with bifunctional peptide-linkers.

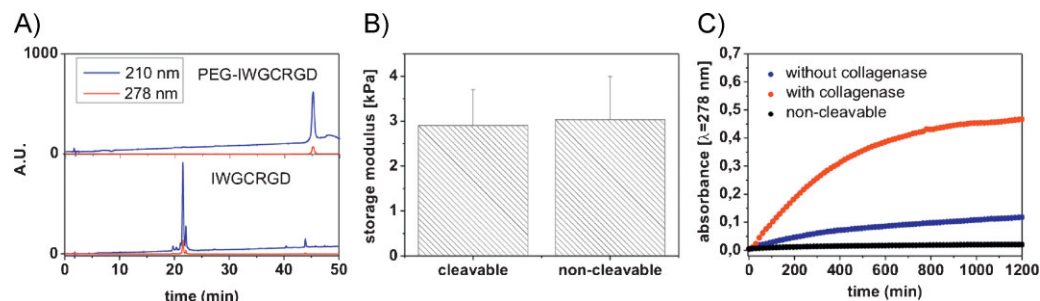


Figure 2. (A) HPLC chromatograms of the MMP-RGD apo-peptide (bottom) and the star-PEG-MMP-RGD conjugate (top); (B) storage modulus of cleavable (starPEG-MMP-RGD-heparin) and non-cleavable (starPEG-heparin) gels with a stoichiometric ratio of 28:08 between the carboxylic groups of heparin and the amino groups of starPEG; (C) enzymatic decomposition of the cleavable and non-cleavable hydrogels in the presence and absence of collagenase IV and PBS, monitored by UV absorption.

groups of starPEG. Gelation occurred within 1 h for all gel types, but the gels were incubated overnight in order to complete the reaction. Thereafter, the hydrogels were swelled in phosphate buffered saline (PBS) for 24 h. Treatment of the cleavable hydrogel with bacterial collagenase IV, a readily accessible model for MMPs,^[28] showed that only the cleavable hydrogel was degraded in the presence of collagenase (Figure 2C). The non-cleavable gel was stable in the collagenase solution for at least a month. The slight increase in the UV-signal for the cleavable gel without collagenase treatment could be related to the nonenzymatic gel degradation upon ester hydrolysis. It has been shown that hydrolysis can occur at 37 °C and pH 7.0 and higher.^[29,30] Nevertheless, the hydrolysis is very slow and takes months for completion, a much longer time scale than is susceptible to cellular processes. Both types of gel were stable in PBS at room temperature for at least a month (data not shown). Additionally, both gels have comparable storage moduli of about 3 kPa (Figure 2B), allowing for the unambiguous comparison of the biofunctional properties of the two-hydrogel systems.

Cell migration is a fundamental process in embryonic development and tissue regeneration in adults. Dynamic ECM remodeling by migrating cells allows three-dimensional cell expansion and tissue growth. Endothelial cells attract special attention among the many cell types which can be used to examine the biocompatibility and proteolytic sensitivity of engineered polymer matrices as angiogenesis, the growth of new blood vessels, is a limiting step in the success of tissue regeneration. As a model system, endothelial cells are known to form three-dimensional tubular structures *in vitro*.^[31] To determine the potential of starPEG-MMP-RGD-heparin hydrogels in tissue regeneration, we investigated the attachment of endothelial cells to the gels and the subsequent cell-mediated reorganization of the materials.

Several *in vitro* studies were performed using primary endothelial cells isolated from human umbilical veins (HUVECs), and the summarized data are shown in Figure 3.

Because cell adhesion regulates the ability and dynamics of cell migration,^[32] cell attachment, spreading, and survival on the engineered surface were initially investigated. Fibronectin (FN)-coated glass, which is widely applied for cell culture studies, was used as a control for cell growth. A non-degradable starPEG-heparin gel, which contains RGD peptides and has similar mechanical properties to the degradable gel (Figure 2B), was used as a control to show the cell response to the presence of MMP-cleavable sequences. After an initial attachment period of only 15 min, cells spread to an equal degree on both gel types suggesting that the MMP-cleavable sequence does not impair cell attachment and spreading, when compared with the non-cleavable control hydrogels.

The projected cell area on both hydrogels was significantly lower than on FN-coated glass which not only has a greater ligand density, but also is a much stiffer substratum (e.g., compare 3 kPa to 96 GPa^[33] (Figure 3A). This was an anticipated result and is in line with literature stating that mechanical properties and ligand density both affect endothelial cell spreading and tube formation.^[34]

After one week of culture, the cell number on the cleavable gels exceeded that of the non-cleavable gels and FN controls, as determined by the colorimetric WST-1 viability/proliferation assay (Figure 3B). Thus, degradable hydrogels allowing for a three-dimensional cell growth (Figure 3C, z-section) can be concluded to serve as a more “natural environment” for endothelial cells than any of the two-dimensional substrates. Moreover, cells growing on the cleavable gels for 7 d showed spindle-shaped morphology, in that they were less spread and formed cord-like structures expanding three-dimensionally. In contrast, cells growing on the non-cleavable gels and FN-coated cover slips grew as confluent, flat monolayers, and were not able to form any significant three-dimensional structures. Furthermore, by loading the hydrogels through heparin binding with vascular endothelial growth factor (VEGF) prior to cell plating, the gels acted as a chemotactic source for the endothelial cells, as indicated by enhanced three-

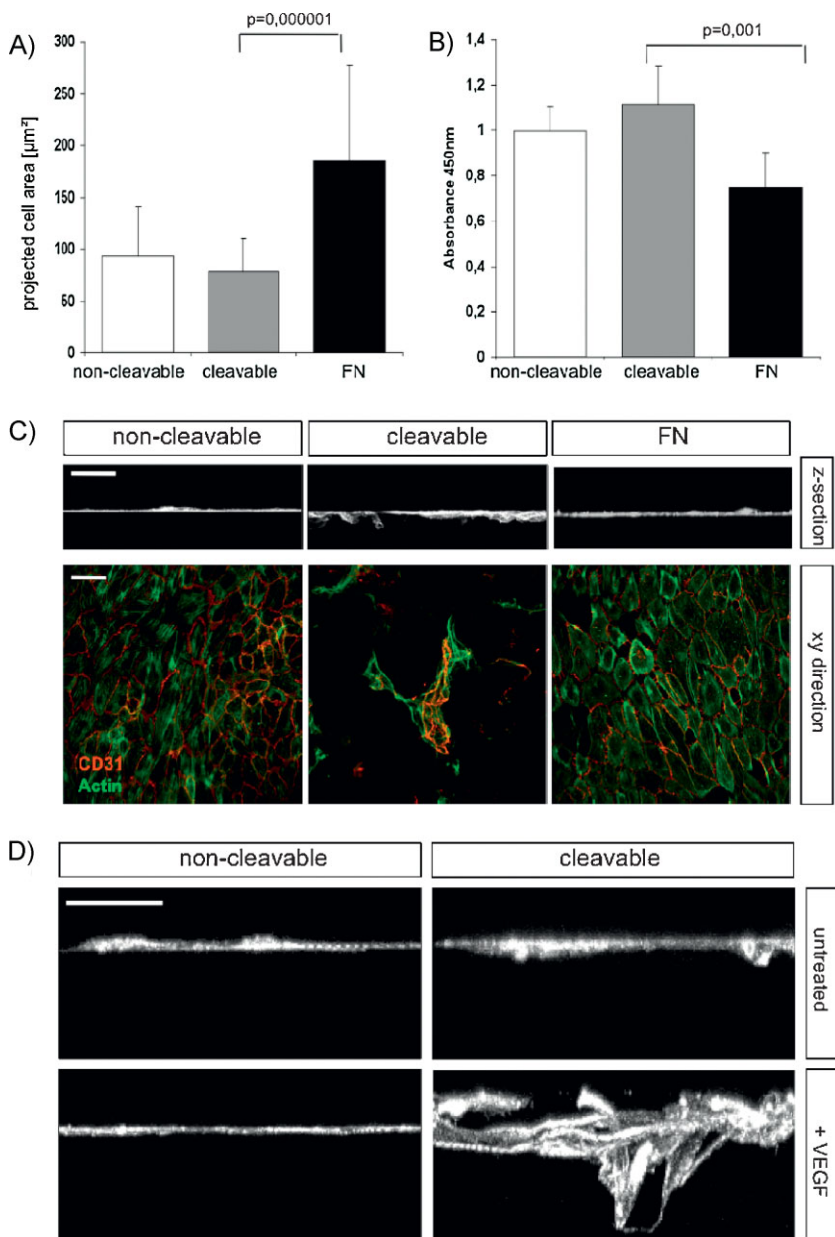


Figure 3. Endothelial cells on different substrates. (A) Cell spreading analyzed by projected cell area (cell number: $n = 30$ on three samples); (B) cell viability/proliferation measured by WST-1 colorimetric assay (samples measured: $n = 6$); (C) representative surface and cross-sectional images of the substrates indicating three-dimensional cell growth within the cleavable gels after 7 d; (D) representative cross-sectional images illustrating enhanced three-dimensional cell migration with VEGF-loaded cleavable hydrogels after 1 d of culture. Length bars = $50 \mu\text{m}$.

dimensional migration into the VEGF-loaded cleavable gels. Cells growing on all three types of surfaces kept their endothelial characteristics, as confirmed by immunostaining with an endothelial-specific membrane marker CD31 (Figure 3C).

The results obtained from the in vitro studies suggest that hydrogel materials containing combined RGD and

MMP peptides support endothelial cells in the formation of three-dimensional structures. Furthermore, by taking advantage of the high affinity of heparin to various signaling molecules, we illustrate that VEGF delivery potentiates endothelial cell chemotactic migration within the reported multifunctional hydrogel materials.

Conclusion

Here, we report recent progress in the development of a modular heparin-starPEG hydrogel platform. Gel materials containing bifunctional peptide linkers with enzymatically cleavable and cell adhesive sequences were synthesized and shown to stimulate three-dimensional growth and matrix remodeling by human endothelial cells. As these are important requirements for pro-angiogenic processes, the gels can be expected to support the vascularization of engineered tissues.

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