Defined Polymer–Peptide Conjugates to Form Cell-Instructive starPEG–Heparin Matrices In Situ

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Extracellular matrices (ECM) are highly hydrated, complex meshworks of proteins and polysaccharides that interactively direct cells in living organisms by orchestrated mechanical and biochemical signals. ECM-inspired polymer hydrogels are being developed and applied to support tissue regeneration in novel therapeutic strategies.[1] In order to allow for the effective, three-dimensional stimulation of cells various recent approaches aimed at forming covalent polymer hydrogel networks in the presence of cells in culture or in vivo, utilizing synthetic polymers as an inert constituent, e.g., poly(ethylene glycol) (PEG) was combined with adhesive peptide ligands or degradable peptide crosslinkers, based on cell-compatible crosslinking schemes, including Michael-type addition,[2] triazole formation,[3] enzymatic crosslinking,[4] and photo-induced polymerization.[5] The Michael-type addition reaction has received special attention as it does not require a catalyst, has no side products, and proceeds rapidly under physiological conditions.[2] The thiol group of cysteine residues can participate as a nucleophile in this reaction which massively extends the number of suitable substrates and simplifies the attachment of bioactive peptides or proteins. A very common synthetic scheme for the formation of PEG-peptide hydrogels utilizes dithiol-containing peptides to react with PEG-polymers carrying electron deficient double bonds at the terminal groups. While this allows for effective hydrogel formation, the resulting materials cannot be independently tuned with respect to contained bioactive components and mechanical characteristics. Moreover, the above mentioned strategies are limited to water soluble components leaving important hydrophobic signal molecules aside.

In alternative schemes, glycosaminoglycan (GAG) conjugates were used instead of dithiol-containing peptides for hydrogel crosslinking.[6] The incorporation of GAGs provides both the protection/sustained release of various growth factors[7] and the precise control of the physical hydrogel properties as GAGs can complex many important factors and serve as multifunctional crosslinkers. However, hydrogels designed according to these schemes were so far neither made to include cell-responsive cleavage sites, nor to decouple the modulation of their different signaling characteristics.[8] To apply the rational design concept recently explored for starPEG-heparin materials obtained through carbodiimide active ester chemistry[8] to similar hydrogels suitable for cell embedding a novel synthetic strategy had to be developed.

Herein, we focused on the development of novel starPEG-peptide conjugates with terminal thiol groups which can be utilized as nucleophiles in Michael addition reactions with electron deficient double bond-containing components (**Scheme 1**). For that we applied a regio-selective amino acid protection strategy for cysteine residues which is commonly used for the synthesis of peptides with multiple disulfide bonds,[9] but has never been utilized for the creation of bioconjugates before. The precise control over the reactivity and specificity of its components allowed us to introduce water insoluble peptides into hydrophilic hydrogel environments, thus overcoming common limitations of dithiol-based Michael-type addition reactions.[10] Using maleimide-functionalized heparin units, we were finally able to apply Michael-type reaction schemes for the in situ formation of customized multicomponent starPEG-peptide-heparin hydrogels with precisely adjusted polymer network properties and independently tunable signaling characteristics. The proposed novel strategy crosslinks heparin-peptide and PEG-peptide conjugates in situ in presence of cell or organ culture, where in particular the modular character of the hydrogel platform allows for the far-going modulation of hydrogel composition and therefore for evaluation of the influence of each component independently or as a combination. As a proof of concept, we show the selective stimulation of embedded human vascular endothelial cells and dorsal root ganglia to undergo morphogenesis into therapeutically desired phenotypes.

To start, various peptide sequences enabling binding of cell adhesion receptors or enzymatic cleavage were conjugated to 4-armed, end-functionalized poly(ethylene glycol) (starPEG). The use of hydrophobic peptides, such as the laminin-derived adhesion peptides (e.g., SIKVAV and YIGSR)[11] is particularly challenging, since the direct incorporation of these peptides in engineered matrix structures is hampered due to their low solubility in water. However, their initial conjugation to multi-armed PEG results in water-soluble, functionalized PEG-peptide building blocks. A well-mixed solution of starPEG and

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DOI: 10.1002/adma.201300691

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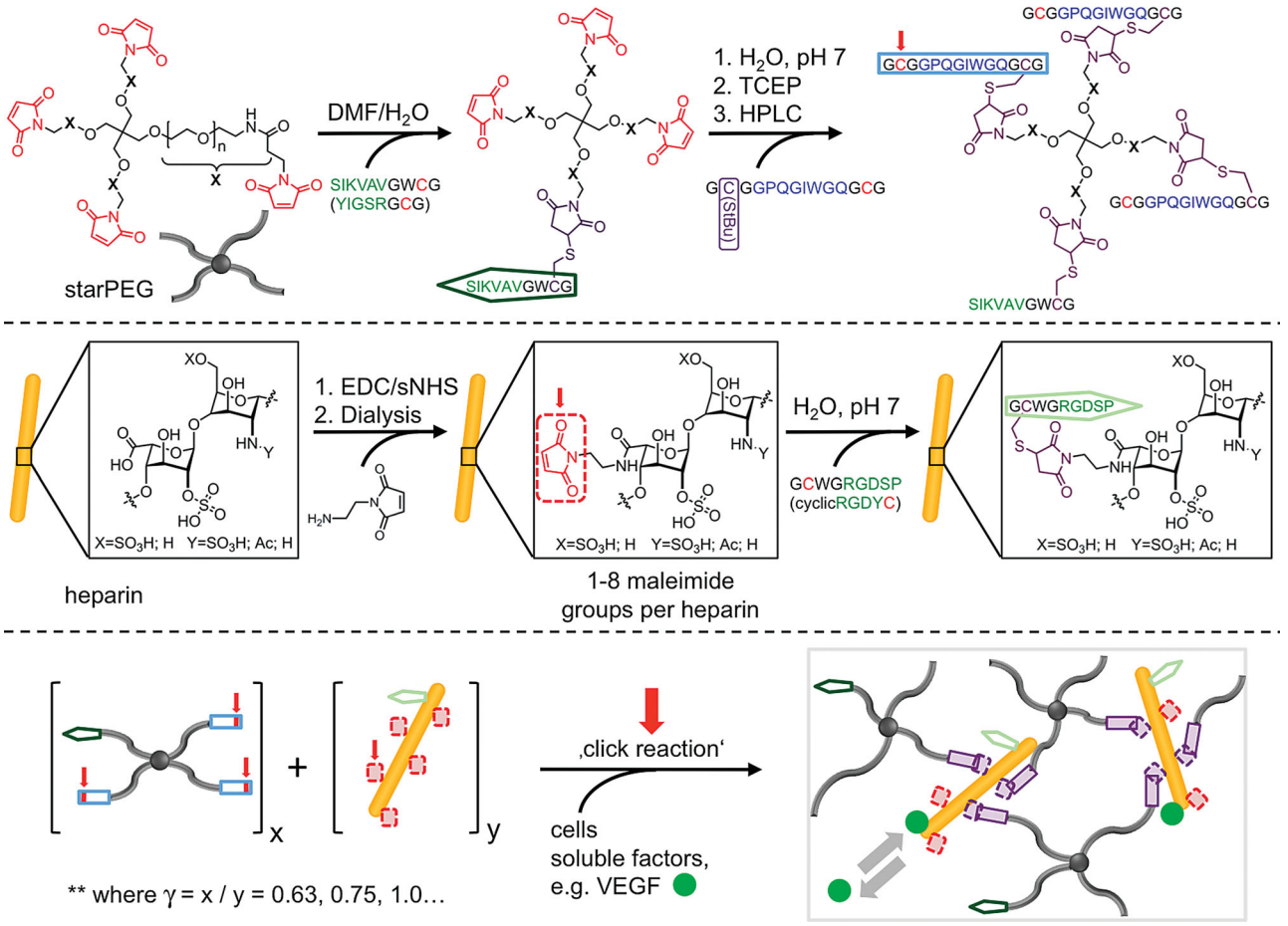

Scheme 1. Synthetic scheme for the formation of multifunctional in situ forming starPEG-heparin hydrogels.

the laminin-derived adhesion peptides SIKVAVGW *C* G and YIGSRG*C* G (where the cell-binding sequence is underlined) was obtained in acidic DMF/H_2O solution. Subsequent elevation of the pH resulted in the fast reaction between the cysteine and maleimide groups to create mono-substituted starPEG-peptide conjugates. Rigorous mixing of the solution prior to the reaction provided a low polydispersity of the formed conjugate: reverse phase high performance liquid chromatography (HPLC) analysis clearly identified the mono-substituted starPEG to be the main product (**Figure 1**A). The obtained starPEG-peptide conjugate was soluble in water and could be further reacted to add additional biologically functional units.

To allow for cell-mediated remodeling of the hydrogel matrix, enzymatically cleavable peptides were pre-conjugated to the remaining maleimide-terminated PEG chains of the SIKVAV- or YIGSR- mono-substituted starPEG to produce a fully substituted starPEG-peptide conjugate. Here, we utilized a matrix metalloproteinase- (MMP-) cleavable sequence as part of a newly designed peptide G*C*GGPQG‖IWGQGG*C*G-NH_2 (enzymatically cleavable sequence underlined)[12] which contains two cysteine residues. The N-terminal cysteine residue was protected with a *tert*-butylsulfanyl (StBu) side chain protection group which is stable during the applied Fmoc strategy-based peptide synthesis and subsequent purification and PEGylation reactions, but is easily removed under moderate conditions (pH 7, room temperature) by reducing agents such as tris(2-carboxyethyl)phosphine (TCEP). This synthetic route effectively prevented the crosslinking of maleimide-terminated PEG with the dithiol-containing peptide avoiding the formation of network defects commonly occurring in other dithiol-based systems. Notably, the use of other thiol region-selective protection concepts such as acetamidomethyl (Asm) or *tert*-butyl (tBu) protection groups required harsher removal conditions (Hg(II), acetic acid/H2O), resulted in side reactions and produced insoluble starPEG-peptide aggregates which significantly decreased the overall yield. The fully substituted starPEG-peptide conjugate was purified by preparative HPLC with an overall yield of >75% and high purity (>99.9% by HPLC) of the final product. Remarkably, the high yield clearly indicates that side reactions, such as the disulfide exchange reaction, have only a minimal impact on the utilized procedure.

The starPEG-peptide conjugates were subsequently crosslinked using commercially available maleimide-terminated starPEG molecules, resulting in enzymatically degradable polymer-peptide hydrogels, or maleimide-functionalized GAG-peptide conjugates (Scheme 1, middle row) to produce GAG-based ternary matrices suitable for further bio-customization. For the second route, we established a method that allows for

DOI: 10.1002/adma.201300691

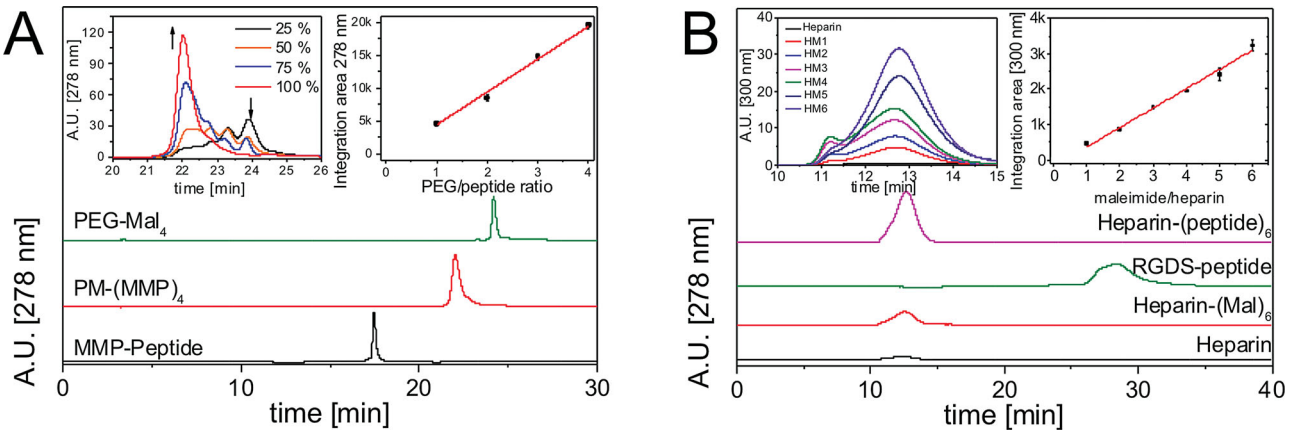

Figure 1. Chromatographic analysis of PEG-peptide and heparin-maleimide conjugates: (A) HPLC chromatogram of purified PEG-$(MMP)_4$ conjugate compared to the precursors MMP-peptide and PEG-$(maleimide)_4$ conjugate:, included subset graphs are the HPLC monitoring of PEG-$(MMP)_3$-SIKVAV formation (left) with the calibration of the peptide stepwise addition by peak integration (right). (B) HPSEC chromatogram of heparin-$(RGDS\text{-}peptide)_6$ conjugate compared to the precursors RGDS-peptide, heparin and heparin-maleimide conjugate; included subset graphs are HPLC monitoring of heparin-maleimide(1–6) conjugate formation (left) with calibration of the amount of maleimide groups per molecule of heparin by peak integration (right).

the attachment of up to six reactive maleimide groups to the backbone of heparin: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfo-succinimide (sNHS) were reacted with heparin creating the sNHS-heparin ester, which reacts stoichiometrically with N-(2-aminoethyl)maleimide to form heparin-maleimide conjugates with well-defined amounts of maleimide groups. Through dialysis under high ionic strength conditions followed by additional dialysis in deionized water, any traces of free maleimides as well as sNHS and hydrolyzed EDC were removed. High performance size exclusion chromatography (HPSEC) was successfully applied for the rapid separation of heparin from any low molecular weight species, such as unreacted maleimide or peptides (Figure 1B). To demonstrate the reactivity of all of the maleimide groups, the purified heparin-maleimide conjugate was mixed at neutral pH with stoichiometric amounts of a cysteine- and tryptophan-containing peptide such as GCWGRGDSP (RGD is a fibronectin derived cell adhesive peptide). The characteristic absorption of the tryptophan residue at 278 nm allows for the quantitative evaluation of the reacted- and free peptide amount in HPSEC, demonstrating the amount of free peptide to be less than 1%. The attachment of peptides to the maleimide-functionalized heparin was precisely adjusted and quantified to make sure that only the desired number of maleimide groups was bound to RGD-peptides, thus allowing for subsequent hydrogel network formation. The demonstrated ultrapurity of the heparin-maleimide conjugate and the availability of a known amount of reactive maleimide groups within its structure can be similarly obtained for other GAG-maleimide conjugates including, for example, hyaluronan or chondroitin sulfate.

To form well-defined hydrogel matrices, thiol-containing starPEG-peptide and maleimide-functionalized heparin were each dissolved in PBS and mixed together at neutral pH, 4% (w/v). Gelation occured within a minute, proving the beneficial reaction speed reported for the maleimide-based Michael-type addition reaction over vinylsulfone or acrylate based reactions.[13] By precisely controlling the ratio of the reactive groups, the crosslinking density and resulting physical properties of the matrix could be tuned independent of the polymer content of the materials: increasing the ratio of starPEG to heparin (γ) at a constant polymer weight percentage lead to hydrogels with a higher storage modulus; therefore, hydrogels of varying stiffness (G' from approx. 200 Pa to 6000 Pa) could be formed (**Figure 2**A). Furthermore, matrices of identical crosslinking degree but different types of cell adhesion sites are all of a similar storage modulus illustrating that the incorporation of different peptides into the hydrogel scheme does not affect the gel properties (Figure 2B).

The obtained MMP-cleavable hydrogels were stable in PBS over time periods exceeding 4 weeks, but rapidly degraded in collagenase-containing solutions (Figure s12), with initial degradation rates that were not dependent on the degree of crosslinking (Figure 2C).

The heparin concentration of the materials remained constant over different crosslinking degrees (Figure 2A) which translates into a similar loading and release of various growth factors (Figure s13) and a constant adhesion ligand density upon conjugation to heparin (Figure s14).

These results prove that the introduced synthetic strategy of combining the defined pre-conjugation of peptide-moieties to starPEG building blocks with the rapid and directed conversion with maleimide-functionalized heparin in fact provides ECM-inspired polymer matrices that allow for the independent modulation of physical and biomolecular characteristics. The short gelation time of the established in situ materials is beneficial for cell encapsulation[13] and facilitates advanced microstructuring approaches.[14]

To demonstrate the suitability of the introduced in situ matrices for triggering cell fate decisions we examined variations of the morphology of primary human umbilical vein endothelial cells (HUVECs) embedded in materials of varying crosslinking densities and biomolecular functionalization (± MMP-degradable peptide linkers, ± adhesive peptides (cyclicRGD) and ± vascular endothelial growth factor (VEGF)) (Figure 2D). StarPEG-MMP-heparin hydrogels conjugated with cRGD were found to allow for cell spreading and nearly 90%

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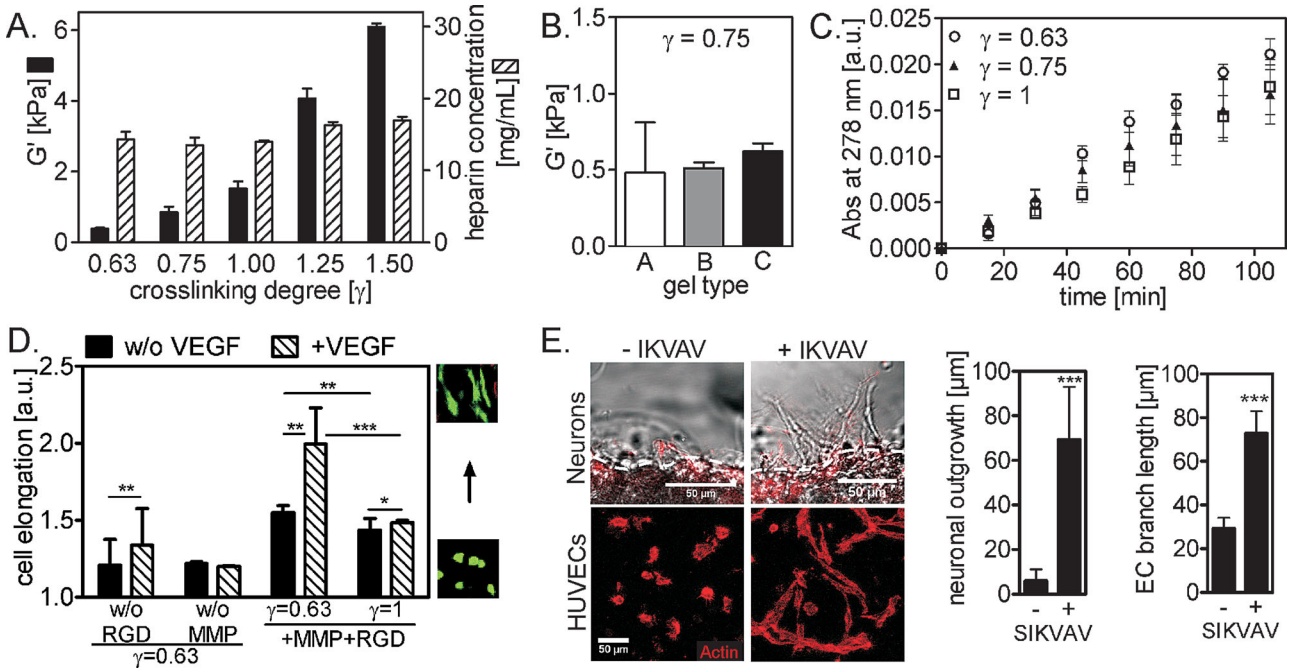

Figure 2. Mechanical properties and biomolecular modification of the hydrogels can be tuned independently (A) The storage modulus (stiffness) (G′) increases with increasing crosslinking (expressed as molar ratio γ of starPEG to heparin) density at invariant heparin concentration. PEG-MMP+2cRGD is shown as a representative example; (B) The stiffness (G′) is independent from the type of the PEG-peptide conjugate: A- MMP-RGD, B- IKVAV-MMP, C- MMP; (C) The initial degradation kinetics is similar for gels with various crosslinking densities, ±SD; (D) Cell elongation (aspect ratio, 1 corresponds to round cell, >1 corresponds to an elongated morphology) in hydrogels is regulated by crosslinking density (γ = 63 vs. γ = 1) and biomolecular modification of the hydrogels (±RGD, ±MMP-peptide, ±VEGF), ±SEM; (E) SIKVAV peptide promotes EC morphogenesis and neurite outgrowth from the DRG, ±SD.

survival of the cells for 24 hours after embedding (Figure s17). Cells were observed to be similarly viable when grown within the gel materials independent of the gel stiffness and presence of VEGF. The overall moderate volume swelling (volume increase approx. 1.3–1.6 times of the initial formed gel for the different crosslinking degrees, see Figure s19) and the resulting low swelling pressure are considered beneficial to maximize cell viability.

Varying the crosslinking densities and biomolecular functionalization had a significant effect on cell morphology. MMP- and RGD-peptide incorporation was indispensable for cell spreading (Figure 2D, s15, s16 and s17). Softer hydrogels ($\gamma = 0.63$, G′≈250 Pa) presenting VEGF were found to induce the most elongated cell shape (Figure 2D) indicating endothelial cell (EC) morphogenesis into a therapeutically desired pro-angiogenic phenotype.

The successful introduction of the hydrophobic, laminin-derived SIKVAV peptide was demonstrated with two in vitro models (Figure 2E): it significantly induced cord-like network formation of ECs and neurite outgrowth (Figure s18) from the dorsal root ganglia, thus showing peptide functionality. Both experiments revealed the importance of the independent control over the different biomolecular signals arising from the successful pre-conjugation to the fully controllable hydrogel matrices. In sum, the presented conjugation methods allowed for the formation of a modular in situ hydrogel platform suitable for displaying multiple adhesion and degradation sites, and growth factors with precisely adjusted network properties. An obvious advantage of the reported peptide-crosslinked starPEG-heparin hydrogels is the incorporation of defined quantities of functional peptide motifs to mediate cell adhesion and cell-demanded matrix degradation. The independent tunability of biophysical characteristics, GAG-concentration, and biochemical modification makes the reported material clearly beneficial over other matrix preparations commonly used in cell biology. The introduced hydrogel could be easily adapted to different cell- or tissue-related requirements thus providing a platform for a variety of advanced in vitro cell culture and in vivo applications in regenerative medicine. In addition, precisely controlled matrices combining non-adhesive PEG polymers, GAGs, multiple hydrophilic and hydrophobic peptidic motives represent a class of multifunctional materials with broad applicability in various different emerging technologies.

Experimental Section

Details of the materials and experimental methods used are available in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Adv. Mater. **2013**,
DOI: 10.1002/adma.201300691

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft through grants SFB-TR 67, WE 2539-7 and FOR/EXC999, by the Leibniz Association (SAW-2011-IPF-2 68) and by the European Union through the Integrated Project ANGIOSCAFF (7th Framework Program). A.Z. was supported by a stipend of the Dresden International Graduate School for Biomedicine and Bioengineering. We thank Marcus Binner (IPF Dresden) for his help in designing the graphics.

Received: February 11, 2013
Published online:

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