Peptides

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Design of Decorin-Based Peptides That Bind to Collagen I and their Potential as Adhesion Moieties in Biomaterials

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Abstract: *Mimicking the binding epitopes of protein–protein interactions by using small peptides is important for generating modular biomimetic systems. A strategy is described for the design of such bioactive peptides without accessible structural data for the targeted interaction, and the effect of incorporating such adhesion peptides in complex biomaterial systems is demonstrated. The highly repetitive structure of decorin was analyzed to identify peptides that are representative of the inner and outer surface, and it was shown that only peptides based on the inner surface of decorin bind to collagen. The peptide with the highest binding affinity for collagen I, LHERHLNNN, served to slow down the diffusion of a conjugated dye in a collagen gel, while its dimer could physically crosslink collagen, thereby enhancing the elastic modulus of the gel by one order of magnitude. These results show the potential of the identified peptides for the design of biomaterials for applications in regenerative medicine.*

An understanding of the key protein–protein interactions involved in cell–matrix interactions, macromolecular organization of the extracellular matrix (ECM), and pathogen-induced and degenerative diseases could form a basis for exploiting this knowledge in biomaterial science and drug design.[1] The task of identifying adhesion moieties to be applied in biomaterials is often conducted by using methods that rely on chance such as phage display[2] or by following a trial and error approach by screening different sequences from ECM components.[3] By contrast, in drug design, a more rational approach using structural information from complexes of the binding partners is the predominant and successful method. Computational analyses and experimental procedures can then be applied for the identification of binding epitopes.[4] Such a method was applied to the rational design of collagen-binding peptides that have relevance to biomaterial applications. Interestingly, despite substantial progress in medicinal chemistry, chemical biology, and biochemistry, biomaterial scientists have so far largely relied on just a few peptide motifs in biomimetic design, such as RGD peptides and enzyme-sensitive peptide sequences.[5] More diverse and specific motifs are required in order to realize novel material functionalities, such as cell-specific binding or molecular structuring of polymers[6], similar to the ECM.[7] The ECM is a hydrogel based on reversible supramolecular interactions and its most abundant protein is collagen. The spatial arrangement of collagen in the ECM is co-organized by decorin, a small leucine-rich repeat (LRR) proteoglycan. Decorin binds to collagen fibrils and controls collagen fibrillogenesis in the ECM,[8] however, the binding mechanism is still not fully understood.[9] In this study, we acquired information on the binding epitope of decorin by rationally developing peptides derived from the two opposite sides of the horseshoe-like 3D structure of decorin, based on the internal repeat structure (Figure 1 A). The peptide with the highest binding affinity for collagen I was then used as a specific adhesion moiety for biomaterials, firstly through conjugation to fluorescein, which was used as a model drug, and secondly for the formation of a physical crosslinker via dimerization. This approach, which involves 1) analyzing and understanding an important biological interaction between two macromolecules, 2) detailing the specific essential epitope and mode of binding,[10] and 3) applying it in the design of a controlled function in materials, is a general strategy for the design of biomimetic materials.

Decorin consists of a core protein, which is composed of 12 tandem leucine-rich repeats (LRRs),[9] each containing an average of 24 (21–30) amino acids. Decorin is functionalized with up to three N-type glycans as well as a single glycosaminoglycan chain close to the N terminus.[11] The decorin crystal structure (pdb entry 1XKU, Figure 1 A, **I**) displays parallel β-sheets on the concave surface (inner surface, IS). The convex surface (outer surface, OS) displays 3_{10} helices, polyproline II helices, and short β-strand or disordered sequences.[12] Homology modeling and docking studies suggested that decorin binds to collagen through the IS[13] and mutations on the inner surface interfere with collagen binding.[14] Interestingly, the IS is also involved in the dimerization of decorin.[15] Owing to the internal repeat structure, it is likely that several repeats are involved in the binding process and enhance the binding affinity through rebinding effects. Our strategy, as depicted in Figure 1 A, was therefore to identify consensus sequences of the IS and OS repeats, to synthesize peptides representing the consensus sequences, and to quantify their binding to collagen in order to determine the binding epitope(s).

Decorin was divided into an IS and an OS on the basis of its crystal structure for a local sequence alignment of the

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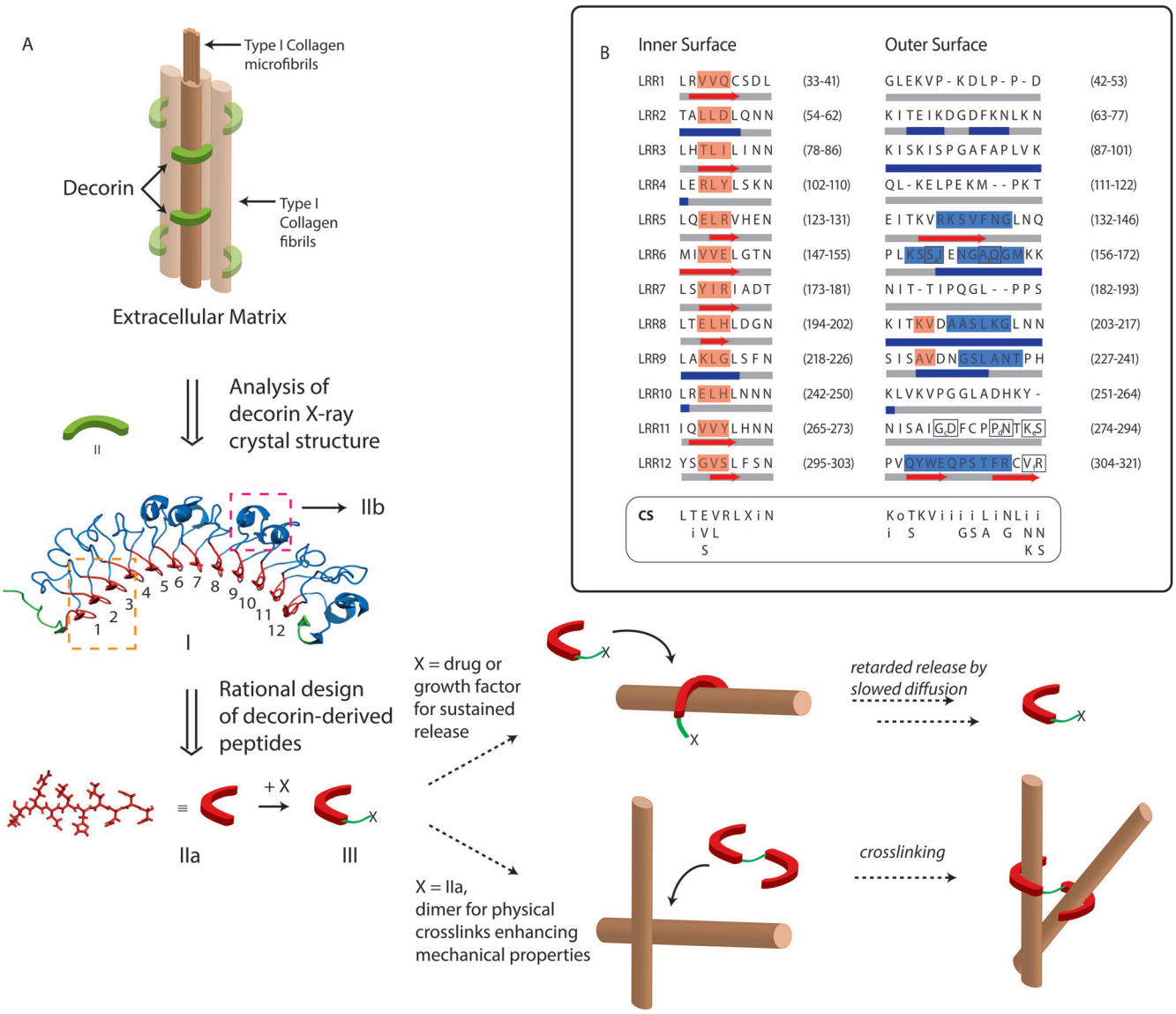

Figure 1. A) Collagen I fibrils in the ECM are organized by decorin (green). Consensus sequences for the IS (red, yellow box) and OS (blue, red box) of decorin (**I**) were proposed. Representative peptides (**IIa**, **IIb**) were synthesized and tested for binding affinity to collagen. A strongly binding sequence (**IIa**) was functionalized with a dye or dimerized (**III**) to create macroscopically observable effects. B) Peptide sequences alignment of the IS and OS repeats. The bars underneath the letters show the predicted secondary structures (red arrows = β-sheets, blue lines = α-helices). The highlighted letters within the sequences correspond to the secondary structures observed in the crystal structure (red = β-sheets, blue = α-helices). Boxes indicate amino acid deletions during alignment for the development of the consensus sequences (CS, bottom).

repeats, while the non-repeat N and C termini were not analyzed.[12] The IS repeat sequences consisted of nine amino acids comprising the major part of the general LRR consensus sequence (**LxxLxLxxN***xL*), which is involved in several protein–protein binding interactions.[16] The OS sequences were 12–21 amino acids in length. A manual alignment of the peptides sequences was performed by categorizing amino acids according to their side chain properties as hydrophobic (V, L, I, F, W, M, C), hydrophilic (G, A, S, T, Q, N, P, Y), acidic (D, E), or basic (K, H, R). This categorization follows observations by Rose et al.[17] but takes potential charges into consideration. Frequency distribution analyses led to the identification of consensus sequences for the IS and OS repeats (Figure 1B). In the manual alignment of the OS repeats, gap insertions in the repeats shorter than 15 amino acids, and amino acid deletions in repeats longer than 15 amino acids (Figure 1B) were made based on the conformation in the crystal structure (Figure 1A, **I**). Peptides representing the consensus sequences (see Table 1) were synthesized by microwave-assisted solid-phase synthesis (Figure 2) using an Fmoc protocol and were purified (purity of all peptides > 95%) by RP-HPLC. The desired mass of each peptide was confirmed by MALDI-TOF-MS. The sequences were characterized by TOCSY, NOESY, and HSQC NMR spectroscopy.[18]

Binding of the peptides to collagen was investigated by surface plasmon resonance (SPR). Varying concentrations of the synthesized peptides or decorin (bovine articular carti-

Table 1: An overview of the synthesized peptides IS 1–6 and OS 1-2, showing predicted secondary structure and dissociation constants (K_D) for binding to immobilized collagen I as determined by SPR experiments.[a]

	Analyte	k_{on} [l mol^{-1} s^{-1}]	k_{off} [s^{-1}]	K_D [M]
	Decorin	2465	8×10^{-6}	3.5×10^{-9}
IO1	LTELRLSNN	n.b.o.	n.b.o.	n.b.o.
IO2	LSELRLHEN	3.0	1×10^{-4}	3.4×10^{-5}
IO3	LTELHLDNN	2.7	4×10^{-4}	1.6×10^{-4}
IO4	LSELRLHNN	22	5×10^{-3}	2.2×10^{-4}
IO5	LSELRLHAN	6.6	1×10^{-4}	1.9×10^{-5}
IO6	LRELHLNNN	15300	3×10^{-3}	1.7×10^{-7}
AO1	KITKVEAASLKGLNN	n.b.o.	n.b.o.	n.b.o.
AO2	KITKIEGAFAKNLNN	n.b.o.	n.b.o.	n.b.o.

[a] Red arrows = β-sheets, blue lines = α-helices. n.b.o.: no binding observed (values rounded independently).

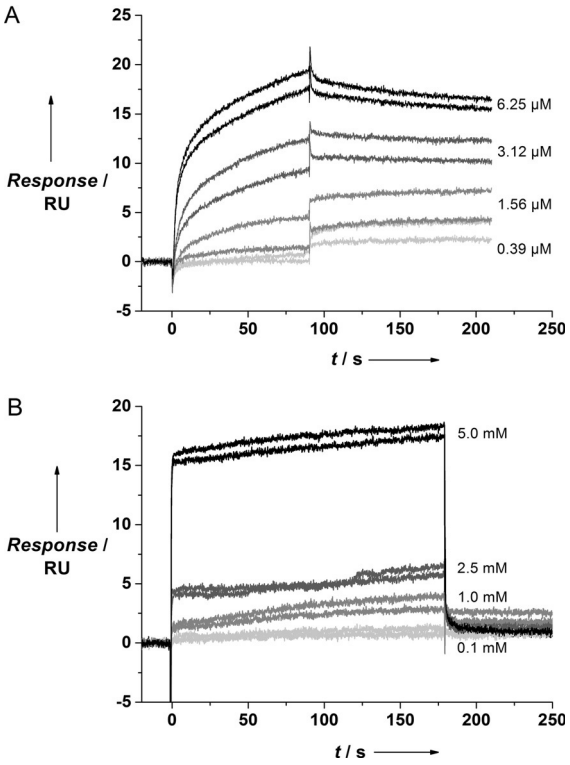

Figure 3. SPR graphs of the binding of decorin (A) and IS-6 (B) to immobilized collagen I indicate concentration-dependent binding.

lage) were passed over a Biacore CM5 chip immobilized with 4000 RU of bovine Collagen I (Figure 3). Quantitative K_D values were obtained by fitting a 1:1 binding model to the curves when analyzing the kinetics ($K_D = k_{off}/k_{on}$). Since several binding sites on collagen with different binding affinities have been proposed for the decorin–collagen interaction, the 1:1 model can only be indicative of an averaged binding affinity and was used in agreement with the literature.[19] The K_D value for decorin and collagen was in the nanomolar range (3.5×10^{-9} M) as reported,[20] while the K_D values for the peptides derived from the IS of decorin ranged from 170 nM to 220 μM (Table 1). The k_{off} values for the peptides were in the range of 5×10^{-3} to 1×10^{-4} s^{-1} but the k_{on} rates were variable. IS-6 showed the lowest K_D value with collagen I. This sequence contains RELH, which has been suggested as a possible complementary sequences to GDRGE, a motif present in the α1 chains of collagens types I, II, and III.[13] An important result of the SPR experiments was that all but one of the peptides based on the IS consensus showed significant binding potential with collagen I, while the two sequences corresponding to the OS of decorin showed no binding.

In a next step, the potential for exploiting the molecular recognition of collagen by the peptides in biomaterial applications was investigated. In a first experiment, a dye conjugate of IS-6, which showed the lowest K_D value of the studied peptides with collagen I, was generated. Propiolated IS-6 (**1**, Figure 2) was coupled to *N*-(5′-azidopentanoyl)-5-aminofluorescein to give **4** by using a copper-catalyzed azide–alkyne cycloaddition reaction. An aqueous solution of **4** was injected into a collagen gel (Figure 4 A). As controls, **4** was injected into a hyaluronic acid (HA) gel and

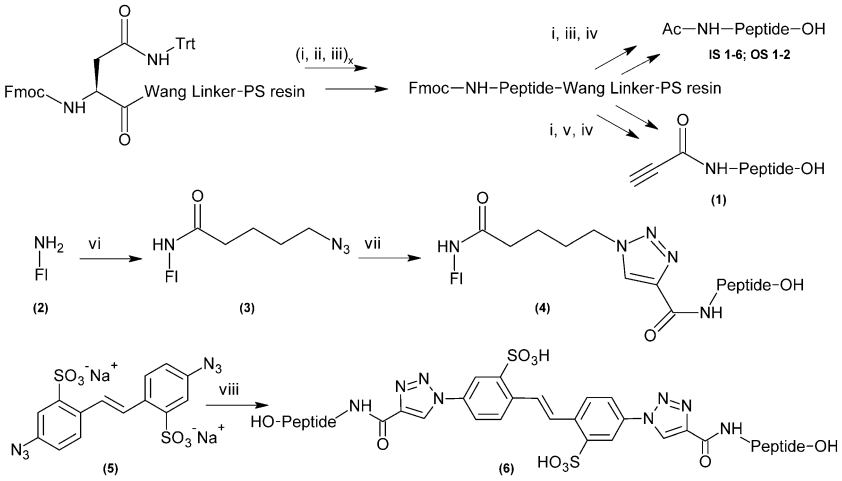

Figure 2. Synthesis of the peptides IS 1-6, OS 1-2, the propionylated peptide **1**, the peptide–fluorescein adduct **4**, and the dimeric peptide **6**. i) 20% (v/v) piperidine in NMP; ii) 10 equiv DIPEA, 5 equiv PyBop, 5 equiv Fmoc-Amino acid-OH; iii) 10% (v/v) Ac_2O in NMP, iv) TFA/TIPS/H_2O 95:2.5:2.5 (v/v); v) 10 equiv DIPEA, 5 equiv PyBop, 5 equiv Propiolic acid, vi) 5-azidopentanoic acid, EDC vii) **1** and CuCl in PBS; viii) 2 equiv **1** and CuCl in PBS. Fmoc = fluorenylmethoxycarbonyl, Trt = trityl, NMP = *N*-Methylpyrrolidone, DIPEA = diisopropylethylamine, PyBop = benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, Fl-NH_2 = 5-aminofluoresceine, TFA = trifluoroacetic Acid, TIPS = triisopropylsilane, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, PBS = phosphate-buffered saline.

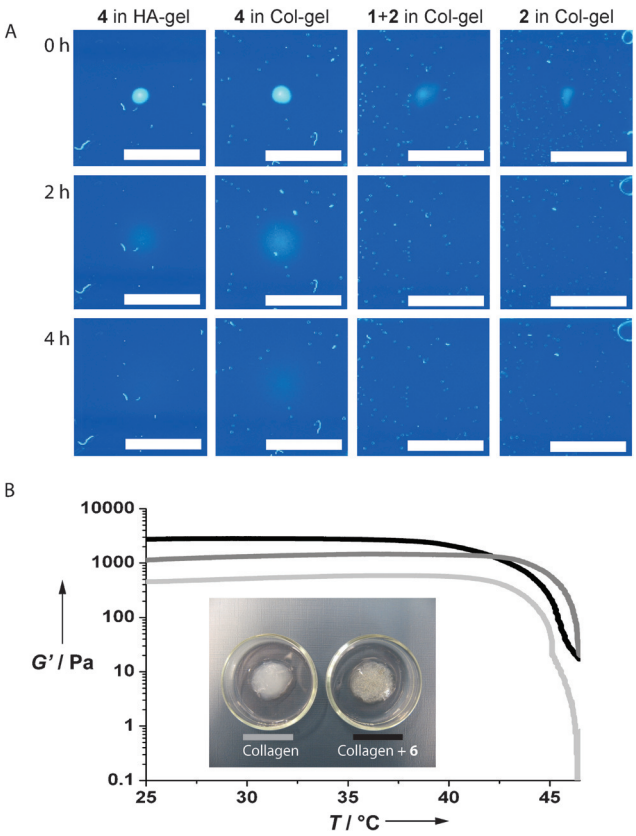

Figure 4. Exploration of the ability of adhesion peptide IS-6 to reduce diffusion in a collagen gel and to enhance the elastic modulus of a collagen gel. A) Diffusion assay in hyaluronic acid (HA) and collagen (CO) gels. **1**: propionylated IS-6, **2**: fluoresceinamine, **4**: propionylated **1** coupled to **2**. Scale bars: 1 cm. B) Rheological behavior of a collagen gel (light gray ■), a collagen gel mixed with propionylated IS-6 (**1**; dark gray ■), and a collagen gel mixed with the IS-6 dimer **6** (black ■). The picture insert shows the CO gel (left, light gray curve) and the CO gel mixed with the dimer (right, black curve). Diameter of the glass dishes = 4 cm.

a physical mixture of the peptide and the dye, as well as the dye alone, were injected into a collagen gel. The diffusion was observed with a Digital Imaging System (UVP). The diffusion of **4** was significantly slower in collagen than in the HA gel and also slower than that of the controls, thus showing that N-terminal functionalization did not hinder biological recognition of collagen by the peptide. In addition, a study of the release of the peptide–dye conjugate from a collagen gel into a water phase demonstrated slower diffusion than that of free fluoresceinamine in a glass chamber model with defined surface areas for exchange processes (see Figure S2 in the Supporting Information). Since both compounds, dye and dye–peptide conjugate, have a molecular weight of less than 2 kDa, which is known to allow free diffusion in collagen gels,[21] the reduced release can be attributed to peptide-mediated binding to the collagen matrix. Therefore, the peptides developed herein might be used for the controlled release of bioactive substances.[22]

Furthermore, it was hypothesized that a peptide dimer could create physical crosslinks between collagen fibrils and enhance the elastic modulus of a collagen gel. To test this, the IS-6 peptide dimer **6** was synthesized by reacting **1** with 4,4′-diazidostilbene-2,2′-disulfonic acid disodium salt (Figure 2). Rheological measurements of collagen gels containing the dimer (Figure 4B) showed increased storage and loss moduli compared to pure collagen gels or collagen mixed with **1**. This can be explained by the peptide dimers acting as crosslinkers between the collagen macromolecules via biological recognition, and this effect could be useful for achieving tailored mechanical properties in hydrogels, as well as for increasing tissue stability. A control experiment (see the Supporting Information, including Figure S1) with the propiolic acid adduct of 4,4′-diazidostilbene-2,2′-disulfonic acid, which lacks the peptide sequence, showed that the aromatic moiety bridging the peptides does not change the rheological behavior of the studied collagen gels. Overall, the results of the rheological experiments support the hypothesis that the enhancement of the mechanical properties of the collagen I gel was based on the effects of the binding peptide sequence.

In summary, the development of consensus sequences for proteins with highly repetitive structures enabled the identification of specific binding peptides with relevance as binding epitopes for protein–protein interactions. These were then exploited to create functional biomaterial systems that exhibit biomimetic biological recognition processes. In addition, experimental evidence for the specific sequences controlling decorin–collagen interactions was generated. The presented strategy for the identification of binding peptides is likely to be most efficient for proteins with highly repetitive structures, however, when structural information on protein complexes is available, the transfer of methods from drug design to biomaterial science will be more feasible. The material functions demonstrated herein, such as adjustment of the mechanical properties of soft materials for tissue replacement or the controlled release of bioactive molecules for prolonged local retention, are highly relevant for applications in regenerative medicine. The general strategy described for the identification of bioactive peptide sequences could provide access to sequences with diverse targets.

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