Biomimetic PEG hydrogels crosslinked with minimal plasmin-sensitive tri-amino acid peptides

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Abstract: Semi-synthetic, proteolytically degradable polymer hydrogels have proven effective as scaffolds to augment bone and skin regeneration in animals. However, high costs due to expensive peptide building blocks pose a significant hurdle towards broad clinical usage of these materials. Here we demonstrate that tri-amino acid peptides bearing lysine (or arginine), flanked by two cysteine residues for crosslinking, are adequate as minimal plasmin-sensitive peptides in poly(ethylene glycol)-based hydrogels formed via Michael-type addition. Substitution of lysine (or arginine) with serine rendered the matrices insensitive to the action of plasmin. This was demonstrated *in vitro* by performing gel degradation experiments in the presence of plasmin (0.1 U/mL), and in the *in vivo* situation of regeneration of critical-sized bone defects. When placed as implants into rat calvaria, gels formed from the minimal plasmin substrates showed clear signs of cell infiltration and gel remodeling that coincided with extensive bone formation. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 93A: 870–877, 2010

Key words: hydrogel; PEG; peptide; plasmin; proteolytic degradation

INTRODUCTION

Tissue regeneration typically starts with the formation of a fibrin hydrogel, polymerized in a wound immediately after injury to stop bleeding, which serves as temporary cellular scaffold for cell infiltration into the wound from the surrounding tissue.[1,2] The fibrin matrix is subsequently degraded and replaced by a newly formed tissue-specific extracellular matrix network. Cell-secreted and -activated proteases such as matrix metalloproteases (MMPs) and the serine protease plasmin are the key effectors in this localized fibrin breakdown. They enable inflammatory and tissue-specific cells to populate the clot and to initiate fibrin remodeling.[3]

Emerging biomaterials approaches to tissue regeneration have focused on mimicking fibrin to form biomaterials *in situ*, at the site of a tissue defect, acting as degradable scaffolds to stimulate the regeneration process.[4,5] The complexity and wide range of targeted clinical applications for such biomaterials necessitates flexible design strategies. The scaffold should have biological and physico–chemical characteristics that are tunable toward specific medical needs and particular tissues. Engineered biomaterials should overcome some of the limitations of "classical," state-of-the-art regeneration matrices such as collagen or fibrin itself. Although these biopolymer networks possess outstanding biological characteristics, their inherent properties such as degradation kinetics are difficult to adapt to specific applications.

Some of us had previously engineered key biochemical characteristics of naturally derived matrices into synthetic, poly(ethylene glycol) (PEG) hydrogel networks.[6–12] Accordingly, hydrogels equipped with peptides to facilitate cell adhesion and susceptibility to degradation by either MMPs,[8–10,13,14] plasmin[7,11] or to both proteases,[12] enable proteolytic cell migration in 3-dimensions, a rather complex physiological

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process. Moreover, in the presence of potent tissue-inducing biomolecules such as bone morphogenetic protein-2 (BMP-2)[8,10–12] or vascular endothelial growth factor,[5,9,15] these hydrogels stimulated extensive bone regeneration and angiogenesis, respectively. The earlier studies also underscored the importance of designing matrix proteolytic sensitivity, as in the absence of this functionality the lack of cell infiltration and matrix remodeling led to significantly reduced tissue regeneration.

Although synthetic biomimetic hydrogels can perform surprisingly well in a biological environment, augmenting bone regeneration or stimulating angiogenesis, they suffer from relatively high production costs when compared with biologically derived scaffolds. Oligopeptide gel components represent the main cost factor for this class of materials, with costs increasing nearly proportional to the peptide length. The high costs can present a limitation to wide-range clinical application.

To reduce the peptide length and, thus, the costs of synthetic biomimetic hydrogels, the main goal of this study was to explore materials designs with minimal length of the proteolytically-sensitive linker peptide. We focused on plasmin-degradable peptides due to the relevance of plasmin in proteolytic remodeling of fibrin[1] and our previous findings with relatively short plasmin substrates as hydrogel components.[11] We found that hydrogels formed with the tri-amino acid peptides bearing either lysine or arginine, but not serine residues, are susceptible to plasmin degradation both *in vitro* and *in vivo*.

EXPERIMENTAL

Materials

Ac-CKC-NH_2 (CKC, M_w: 394 Da, net peptide content: 70.6%), Ac-CRC-NH_2 (CRC, M_w: 422 Da, net peptide content: 73.8%), and Ac-CSC-NH_2 (CSC, M_w: 353 Da, net peptide content was not provided and was assumed to be 100%) were purchased from Neosystems (Strasbourg, France). These peptides were designed containing single amino acids flanked only by cysteines to allow the peptides to undergo crosslinking with branched, vinylsulfone (VS)-terminated PEGs to form hydrogels via Michael-type addition. Peptide purity was 95–96% as determined by HPLC analysis. Peptide Ac-GCYK↓NR↓DCG-NH_2 (C2KR, M_w: 1056 Da; ↓ marks cleavage sites) was synthesized and purified as reported earlier.[11] A 4-arm PEG-OH (M_w: 20 kDa) was purchased from Nektar (Huntsville) and functionalized with VS groups PEG (4-arm PEG-VS) as described earlier.[16,17] The degree of end-group conversion assessed by ^{1}H NMR (400.13 MHz, $CDCl_3$) was shown to be 81%: δ 6.87–6.78 (dd, CH=CH_2), 6.43–6.36 (d, CH=CH_2), 6.10–6.06 (d, CH=CH_2), and 3.69–3.61 (s, CH_2CH_2O).

Rheometry and equilibrium swelling (Q_m)

Rheological experiments were performed with a Bohlin CVO 120 high-resolution rheometer with plate-plate geometry at 25°C as described previously.[16] Q_m was defined as the ratio of the mass of swollen gel to the mass of dried gel.

Hydrogel formation

Hydrogels were formed at 20% (w/v) via Michael-type conjugate addition of peptide thiols (Schemes 1 and 2) onto VS of end-functionalized 4-arm PEG-VS in 0.3*M* of triethanolamine (TEOA) buffer at pH 7.6, using previously published methods.[16,17] Briefly, to form 25 μL hydrogels containing 20% (w/v) polymer, precursor solutions were mixed at various stoichiometric ratios r = [SH]/[VS] (SH, cysteine thiols; VS groups on PEG termini) and formed as disks between two Sigma-coated (Sigamacote®, Sigma-Aldrich, Germany) glass slides separated by 0.8-mm thick spacers. Crosslinking was allowed to proceed for 30 min at 37°C in a humidified atmosphere, and the gels were swollen overnight in 0.1*M* phosphate buffered saline (PBS) (pH 7.4).

Hydrogel degradation

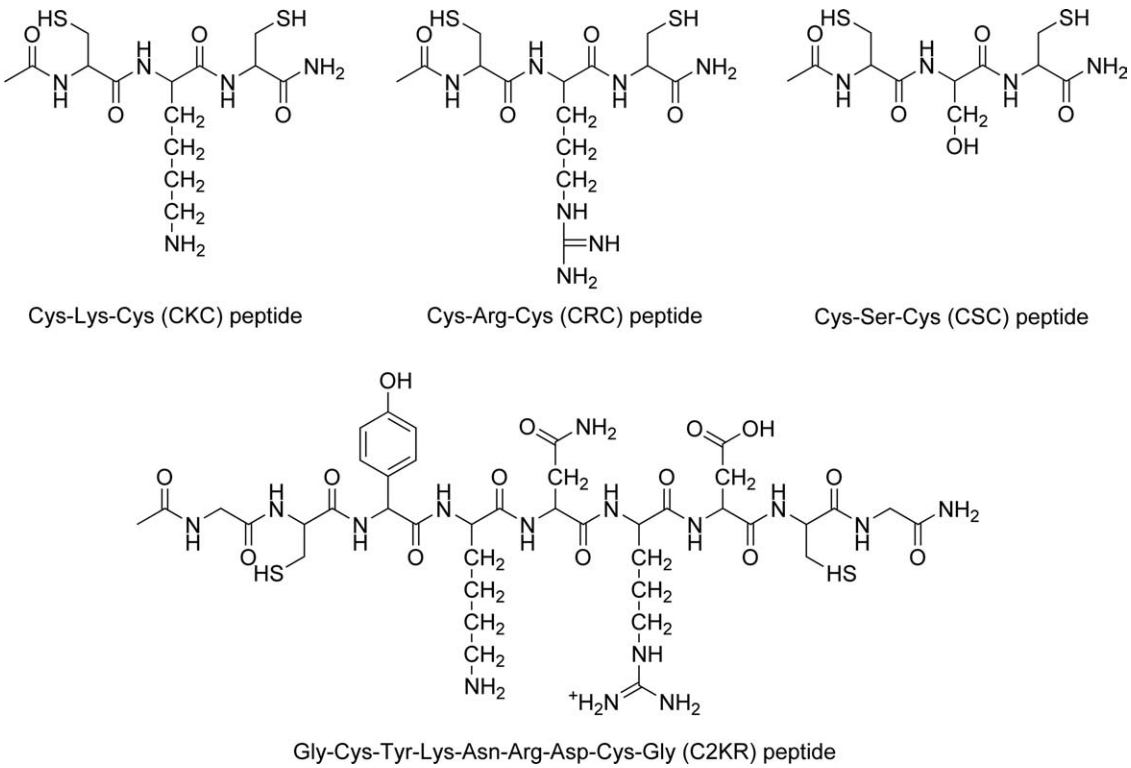
Hydrogel disks (25 μL) were incubated in a solution of 4-mL PBS containing 0.1-U/mL bovine plasmin (Roche, Switzerland). Samples were weighted at different time points. Each condition was measured in triplicate.

Preparation of hydrogel disks for animal experiments

To facilitate cell invasion into nonadhesive hydrogels, gel formulations described earlier (see Hydrogel formation) were augmented to covalently attach the integrin-binding peptide Ac-GCGYG*RGDSP*G-NH_2 to the gel network following published protocols.[10] Moreover, 5 μg rhBMP-2 was physically entrapped into gels by mixing it with the PEG precursor before gelation, according to published methods.[10]

Animal experimentation

Animal experiments were authorized by the Veterinary Authority of the Canton of Zurich. Adult female Sprague-Dawley albino rats (300–350 g) were used and bone regeneration experiments were conducted exactly according to published methods.[10,11] Explants were sequentially dried, defatted, fixed, and embedded. Sections were stained with Toluidine blue O and Goldner Trichrome. Three animals were used per gel type.



Scheme 1. Designed tri-amino acid peptides (top). The plasmin-sensitive peptide C2KR (bottom) was selected as a positive control based on earlier work.[11]

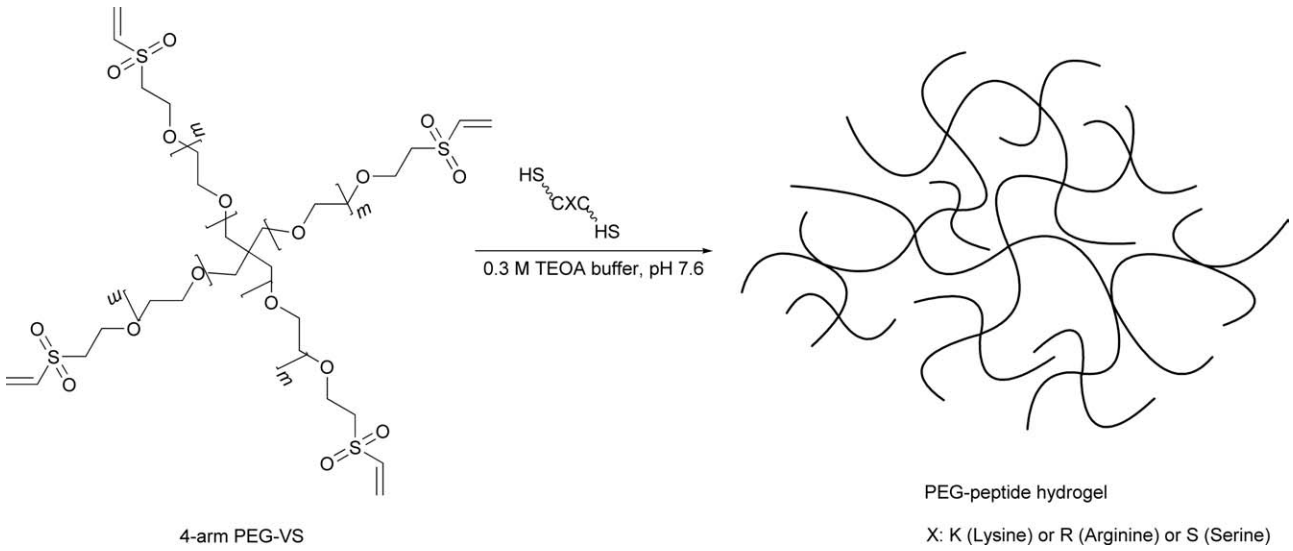
RESULTS AND DISCUSSION

Design of minimal putative plasmin substrate peptides

West and Hubbell had previously synthesized PEG-based gels from telechelic macromers modified by the plasmin-sensitive oligopeptide Val-Arg-Asn (VR↓N, ↓ denotes proteolytic cleavage site), derived from the α-chain of fibrinogen. This peptide was found to be cleavable by plasmin proteolysis, yet insensitive to the action of other proteolytic enzymes, such as MMPs or elastase.[6] In later generations of PEG-based plasmin-sensitive hydrogels, some of us used either single or multiple fibrinogen-derived plasmin substrates within a given peptide, Ac-GCYK↓NR↓DCGYK↓NR↓CG-NH_2,[11] or recombinant protein polymer, -(...NNR↓DNTYNR↓VSEDL...)$_n$-,[7] -(...NNR↓DNTY...)$_n$-[17] Interestingly, in the latter study, biochemical investigations revealed the presence of unexpected plasmin cleavage sites in negative control constructs and N-terminal sequencing of plasmin-degraded products indicated that the C-terminus of arginine residues in the proteins might act as substrates for plasmin.[17] Indeed, arginines and lysines typically mark cleavage sites in proteins[18] and are commonly known P1 sites (i.e., amino acids adjacent to the C-terminus of the proteolytic cleavage site). Oftentimes, arginine cleavage sites are flanked by amino acids with aromatic side groups such as tyrosine or tryptophan.[19,20] Based on these findings, we hypothesized that isolated lysine or arginine residues might be sufficient to act as substrates for plasmin in PEG-based hydrogels. To test this hypothesis, we designed peptides containing single amino acids flanked only by cysteines (cysteine-lysine-cysteine, CKC, and cysteine-arginine-cysteine, CRC, see Scheme 1). This peptide design allowed the peptides to undergo crosslinking with branched, VS-terminated PEGs to form hydrogels via Michael-type addition (Scheme 2). As potential negative control peptide, the amino acid serine was flanked by cysteines.

Hydrogel formation and characterization

We next tried to form solid hydrogels via Michael-type addition (Scheme 2) by mixing two liquid precursors containing 4-arm PEG-VS and each of the above Cys-containing tri-amino acid peptides (Scheme 1). Indeed, at pH 7.6, we were able to form hydrogels within about 10 min after mixing of relatively highly concentrated aqueous precursors. Remarkably, when compared with our previous PEG-co-peptide gel system,[16] in which networks were formed at precursor concentrations as low as 5% (note: same PEG macromers were used but 16-



Scheme 2. PEG-co-peptide hydrogel formation by Michael-type addition.

amino acid-long peptides), gel crosslinked with very small peptides only formed reliably at concentrations above 15%. To determine the ratio of functional groups that would lead to nearly ideal gel networks,[16,17] the equilibrium swelling ratio Q_m and elastic modulus G' of swollen gels were determined as a function of r = [SH]/[VS] (Figs. 1 and 2). A minimal Q_m and maximum in G' at r_{opt} was found at slightly excessive amounts of SH-groups for CKC (r_{opt} = 1.1) and CRC (1.15), respectively, and r = 0.9 for CSC.

The slight excess of thiols that was required to obtain optimal physico–chemical properties in CKC and CRC gels can most likely be attributed to the SH-consumption via disulfide bond (—S—S—) formation, that may occur concurrently with the Michael-type addition.[16,17] The rather unexpected r_{opt} of hydrogels crosslinked with CSC ($r < 1$) may be explained by a different reactivity of thiols to form either covalent bonds with VS or disulfide bonds due to differences in thiol pKa's.[21] This is to say, we had previously demonstrated that the pKa of the sulfhydryl groups of cysteines is highly dependent on adjacent charged amino acids, positively charged amino acids lowering the pKa, and negatively charged amino acids having the opposite effect. Because deprotonated thiols are the reactive species in the conjugate addition reaction, lysine and arginine render thiols in CKC and CRC more reactive than serine in CSC.

Hydrogel degradation characteristics

To investigate the proteolytic degradation kinetics upon exposure to plasmin, hydrogels with different linker types but equivalent swelling ratios and elastic moduli (r = 0.85 for CSC, 1.1 for CKC, and 1.15 for CRC, respectively) were selected, and swelling and weight loss determined as a function of incubation time (Fig. 3). As expected, gels containing the plasmin-sensitive control peptides C2KR showed the fastest degradation, primarily mediated by surface erosion (i.e., no increase in gel weight during dissolution). Complete gel dissolution occurred after about 5 h of incubation, consistent with previous findings[11] [Fig. 3(a)]. In marked contrast, no weight change and thus degradation was observed during the 6-day test period in gels containing the control peptide CSC.

CKC- and CRC-containing hydrogels exhibited sensitivity to plasmin degradation [Fig. 3(a,b)]. CKC-bearing gels degraded significantly faster than those crosslinked with CRC (ca. 130 h vs. ca. 240 h for complete gel dissolution, respectively). We also noted that CKC- and CRC-gels initially increased in weight during degradation, suggesting a predominant bulk degradation mechanism, in contrast to C2KR-containing control gels that appeared to primarily degrade via surface erosion. Notably, the peptide VRN (Val-Arg-Asn) that was introduced in earlier work as plasmin-sensitive substrate within photopolymerized PEG-based hydrogels showed slower degradation kinetics (400 h at 2 U/mL plasmin; 600 h at 0.2 U/mL)[6] when compared with gels made with CKC-peptide (130 h at 0.1 U/mL plasmin). Therefore, these results clearly demonstrate that arginine or lysine alone, when flanked by reactive cysteines, can serve as effective plasmin substrates in crosslinked PEG-based hydrogels. However, it should also be noted that other proteolytic enzymes, such as MMPs, may cleave these peptides.

Proteolytic enzymes usually bind to substrates comprised of several amino acids. Various methodologies, ranging from classical amino acid sequence studies[22] to positional scanning libraries of fluoro-

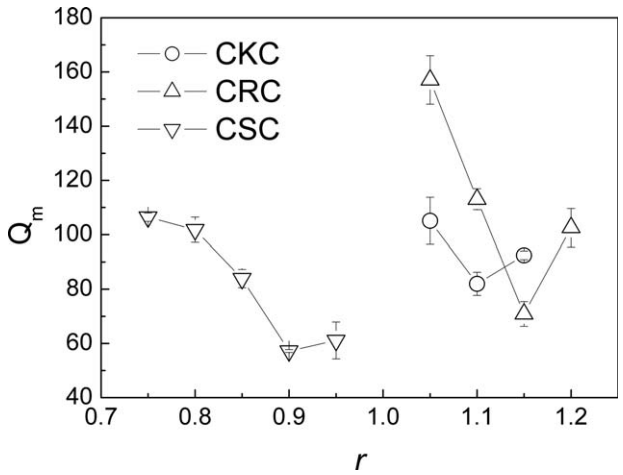


Figure 1. Equilibrium swelling (Q_m) of hydrogels formed from 4-arm PEG-VS and tri-amino acid linker peptide at different stoichiometric ratios r = [SH]/[VS]. (Error bars: Standard error of the mean, n = 3).

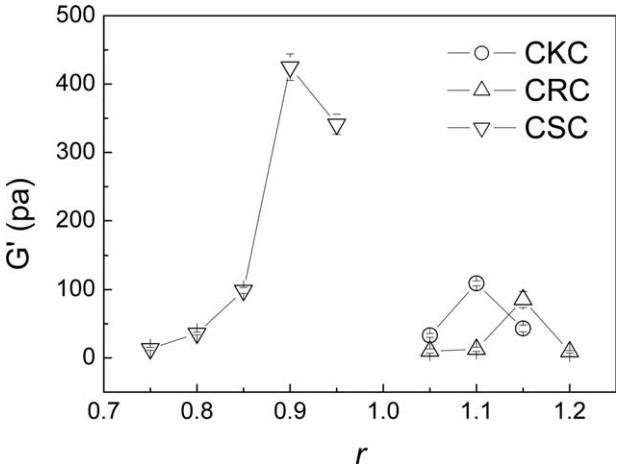


Figure 2. Elastic shear modulus (G') of hydrogels formed from 4-arm PEG-VS and tri-amino acid linker peptide at different stoichiometric ratios r = [SH]/[VS]. (Error bars: Standard error of the mean, n = 3).

genic peptides[19] and microarray techniques[20] have been used to uncover the primary peptide structure of fibrinogen plasmin substrates. Specificity is primarily determined by the amino acid in P1 position. In some substrates, the P1 residue and the following P1′ amino acid will contribute to interactions that define the cleavage site.[23] It is widely accepted that substrates with lysine at P1 are degraded faster than with arginine in that position,[19,20,24] which is in agreement with our results. From structural analyses of the human fibrinogen α-chain, several amino acids including methionine, serine, or alanine were identified as P1′ sites,[22] though it is still poorly understood which P1′ amino acid induces the fastest degradation.[25] Moreover, the active site of plasmin fits best in a substrate "pocket" that contains tyrosine or tryptophan at P2.[19] The C2KR peptide fits these criteria and shows significantly faster degradation when compared with CKC or CRC. To the best of our knowledge, polymer-conjugated cysteines were not previously reported as P1′ and P2 sites. Our results show that cysteines with their thiols bound to VSs via thioether bonds can act as P1′ and P2 site, as plasmin degradability of gels bearing Cys-containing tri-amino acid peptide is retained.

In vivo enzymatic degradation: Induction of bone regeneration

To test the susceptibility to degradation of the newly designed plasmin-sensitive gels in a relevant *in vivo* scenario, we utilized the previously established rat calvarial bone defect model.[26] This model is frequently used to test the suitability of bioactive molecules, such as BMP, and scaffolds to induce regeneration of large bone defects and our group had used it successfully in the development of plasmin-sensitive gels.[11] We fabricated gel disks (8 mm diameter) containing noncovalently entrapped BMP-2, implanted the samples in critical size defects of

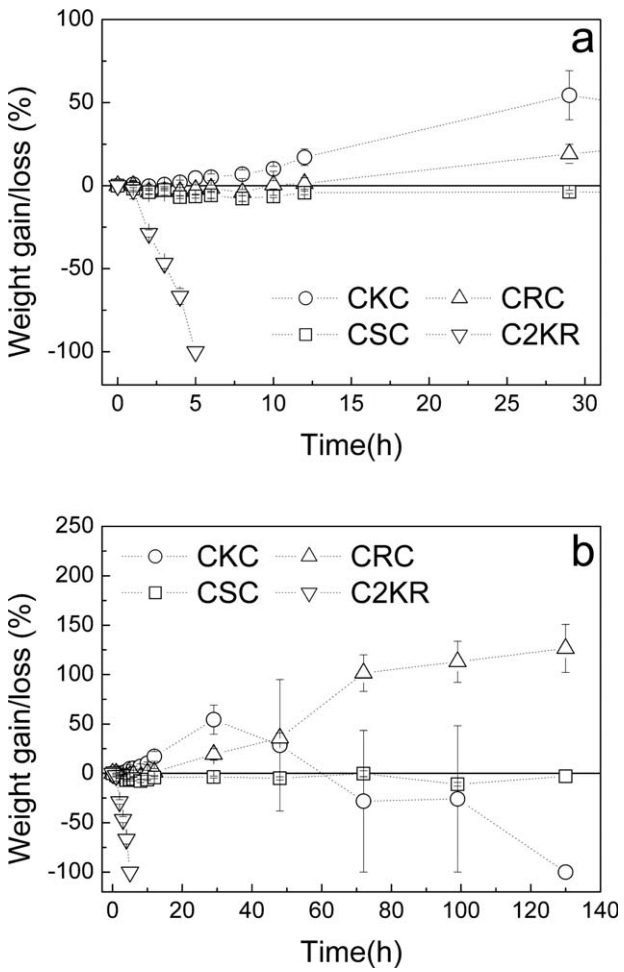


Figure 3. Degradation of hydrogels in PBS containing 0.1 U/mL plasmin at 37°C monitored for 30 h (a) and 130 h (b). (Error bars: Standard error of the mean, n = 3).

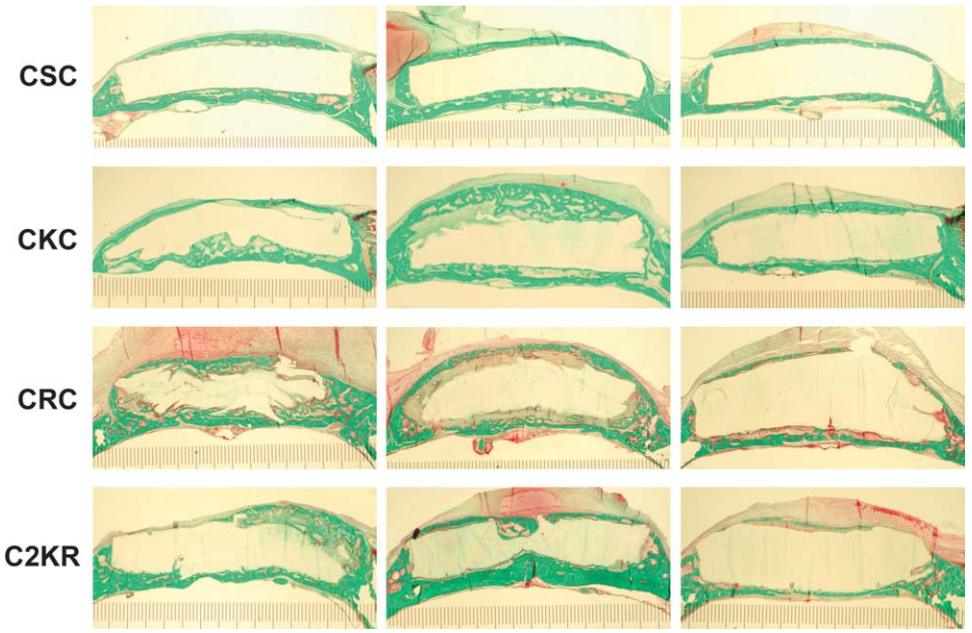


Figure 4. Histological analyses of cell infiltration and bone healing. Cross-sections of defects 3 weeks after gel implantation. Representative sections of implants of three animals are shown for each gel type.

the same diameter in rats, and sacrificed animals after 3 week to analyze via histology the response of the materials to its *in vivo* environment. Histology demonstrated a highly materials-dependent cellular infiltration and bone-healing outcome (Fig. 4).

Nondegradable CSC-type gels showed a sharp interface between the still intact hydrogel and the surrounding tissue (Fig. 4, top row; note that the mineralized phase shows up in green). Apparently, the presence of 5 μg BMP-2 per implant led to bone formation

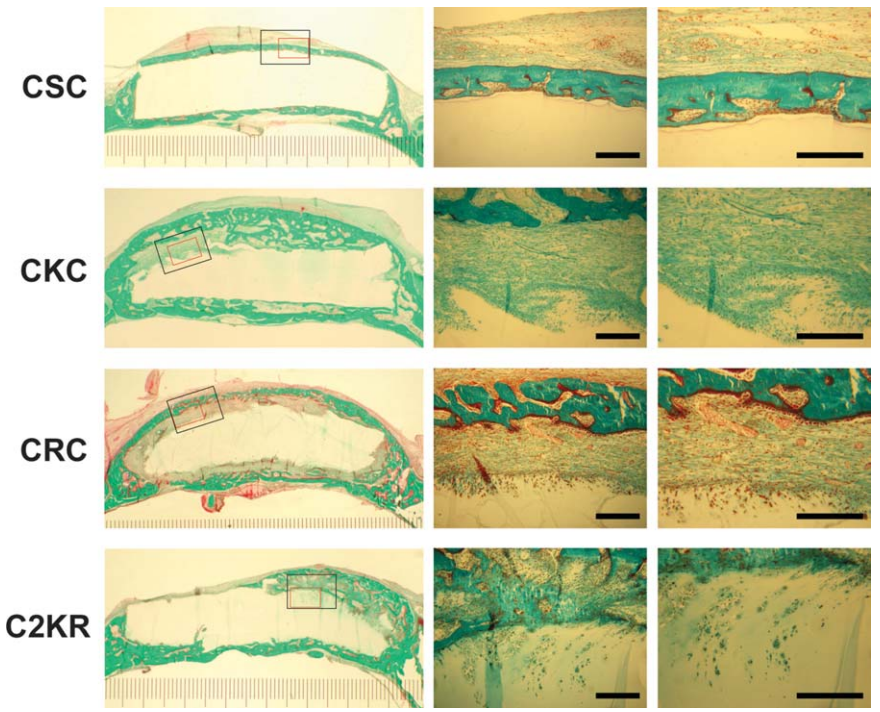


Figure 5. Histological analyses of cell infiltration at higher magnification. Cross-sections of defects 3 weeks after gel implantation. Representative sections of one implant are shown for each gel type. The small black and red squares in the complete cross-sections correspond to the higher magnification shown in the middle and right columns, respectively. (Scale bars = 200 μm).

around the plasmin-insensitive implants. The smooth surface of the gel phase indicated that no cell infiltration took place. Therefore, the nondegradable and relatively dense gel matrix acted as a physical barrier for surrounding cells. Qualitatively, plasmin-degradable gels showed a markedly different outcome. The tissue-gel interface of most explants was not smooth but rather irregularly shaped, showing signs of extensive infiltration by host cells (Fig. 4).

As is evident at higher magnification (Fig. 5), these irregularities were created by local, cell-mediated gel breakdown, consistent with earlier findings.[11] In some implants, the plasmin degradation of hydrogels enabled a more pronounced *de novo* bone formation through gel remodeling, when compared with the nondegradable control. Surprisingly, the most extensive gel degradation was observed in CRC gels and not in C2KR gels that *in vitro* showed the fastest degradation (Fig. 3). The apparent contradiction between *in vivo* and *in vitro* data may be attributed to the fact that in a complex *in vivo* regeneration microenvironment, many types of proteases can be found and therefore, even though the peptides were designed as plasmin substrates, other proteases such as MMPs could be responsible for the *in vivo* gel degradation. Moreover, due to the variability among the animals of even the same gel type, which may be attributed for example to differences in mechanical properties but also differential BMP2-retention (previously shown to be ca. 90% in PEG-based gels[10]), larger sample numbers and a quantification of healing are crucial to substantiate these data.

In an earlier study of gels composed of the same plasmin substrate YKNRD as in the present C2KR gels, a more extensive cell infiltration was observed at 3 week and the same dose of BMP-2.[11] In some regions, the entire thickness of implants was replaced by cellular processes. This discrepancy may be explained by the fact that the previous gels were composed of a different molecular architecture (peptides containing three cysteines crosslinked with linear PEG-VSs) that led to a less densely crosslinked network, which could have resulted in a more rapid degradation of implants. Nevertheless, our experiments revealed that minimal plasmin substrates as gel components can be degraded via endogenous mechanisms in an *in vivo* context, and in which this characteristic can be explored to regenerate large bone defects in a critical size defect model. Longer implantation times will reveal whether these novel gels are completely remodeled by natural mineralized tissue.

CONCLUSIONS

We show that tri-amino acid peptides bearing lysine or arginine, flanked by cysteines, can serve as key functional components of plasmin-degradable PEG hydrogels. Substitution of lysine or arginine with serine residues rendered the hydrogels insensitive to plasmin. Despite of the slower *in vitro* gel degradation rate when compared with gels with longer, naturally derived plasmin substrates (C2KR), in an *in vivo* context of bone regeneration, the maximally truncated substrates performed similarly well. The modularity to incorporate desired biomolecules into the present hybrid hydrogels, together with their significantly lower costs, promises to render these materials attractive for clinical applications.

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