Published in final edited form as:
Biomaterials. 2007 December ; 28(35): 5215–5224.

Facile coupling of synthetic peptides and peptide-polymer conjugates to cartilage via transglutaminase enzyme

Marsha Ritter Jones[1] and **Phillip Messersmith**[1,2,3]

1 *Biomedical Engineering, Northwestern University, Evanston, IL, USA*

2 *Materials Science and Engineering, Northwestern University, Evanston, IL, USA*

3 *Institute for Bionanotechnology in Medicine, Northwestern University, Evanston, IL, USA*

Keywords

transglutaminase; cartilage; peptide; polymer; conjugation; surface

Introduction

Strategies for chemically coupling natural or synthetic molecules to biological surfaces are important tools for drug delivery, tissue repair, and fixation of tissue engineered scaffolds for tissue regeneration[1]. Several methods are capable of attenuating, inhibiting or promoting interactions between tissue surfaces as well as between the cells and extracellular matrix (ECM) proteins that comprise them. Electrostatic interactions have been employed by Elbert *et al.* in the form of poly-L-lysine-graft-(poly(ethylene glycol) polymers that chemisorb to proteins on tissue surfaces, and this approach was explored as a strategy to minimize postsurgical adhesions [2]. Winblade *et al*. employed phenylboronic acid modified polymers[3,4] to provide reversible covalent crosslinks to *cis*-diols in sugar residues of glycoproteins and polysaccharides. Layer by layer (LbL) assembly of polyelectrolytes has been used to apply polymer coatings onto model biological surfaces[5], the surface of blood vessels[6] and pancreatic islets[7].

Specific functional groups found in ECM proteins have been exploited for covalent surface modification strategies. For example, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) have been used extensively to couple macromolecules containing carboxylic acids to protein amines[8], [9], [10]. Amine-reactive PEG diisocyanates have been used to modify pancreatic islets in order to provide immunoprotection[9,11], to create a barrier to platelet adhesion on damaged arteries[12,13], and on preclotted Dacron and other model biological surfaces[14]. Aldehyde modified chondroitin sulfate, which also reacts with tissue amines, has been used as a tissue adhesive in both the cornea[15,16] and cartilage [17,18]. Photochemical oxidation of native tyrosine residues in collagen II has been used to improve the integration of photopolymerized hydrogels with cartilage[19].

Corresponding Author: Phillip B. Messersmith, Biomedical Engineering Department, Northwestern University, 2145 Sheridan Rd, Evanston, IL 60208, TEL 847-467-5273, FAX 847-491-4928, philm@northwestern.edu.

Supporting Information
Figure S1 and MALDI-TOF mass spectrums of all peptides synthesized.

In contrast to chemical or photochemical approaches, we seek to develop a general strategy for tissue surface modification that relies instead on biological enzyme mediated crosslinking reactions. Transglutaminases (TG) are calcium-dependent enzymes that catalyze crosslinking between lysine and glutamine residues to form ε-(γ-glutaminyl) lysine isopeptide bonds[20]. There is growing interest in the use of transglutaminase enzymes for tissue repair and reconstruction. Calcium-independent microbial TG[21] has been used to develop gelatin hydrogels for biomedical adhesives[22] as well as for *in vitro* expansion of cells[23]. Factor XIII, the circulatory form of TG, has been used to form fibrin matrices for *in vitro* and *in vivo* studies of neurite growth[24], angiogenesis[25,26], and cartilage regeneration[27–29]. The incorporation of bioactive peptides and proteins into these matrices was achieved by including a Factor XIII reactive peptide domain within the molecule[30–32]. Synthetic polymers have also been modified with Factor XIII substrate peptides, which were then crosslinked by the enzyme into a hydrogel[33].

A transglutaminase enzyme found in many connective tissues and often referred to as tissue transglutaminase (tTG) was used by Sperinde and Griffith to form hydrogels through crosslinking of glutamine modified poly(ethylene glycol) (PEG) polymers and a lysine containing polyaminoacid[34]. Hu *et al.* subsequently employed rationally designed peptide substrates of tTG to modify PEG polymers to form an adhesive hydrogel[35,36], and tTG was recently used to crosslink an elastin-like polymer (ELP) coacervate that contained lysine and glutamine residues[37]. Finally, tTG has also been used to couple biomolecules to insoluble peptide assemblies[38], and was used to enhance cell adhesion and spreading on collagen matrices and synthetic polymers coated with fibronectin[39,40].

tTG has attractive features for enzymatic coupling of molecules to tissue surfaces because it operates under mild physiologic conditions and has a number of known protein substrates found in the ECM[41,42] and on cell surfaces[43]. In this paper, we explore the use of (tTG) enzyme as a simple and biocompatible method for coupling synthetic molecules to tissue surfaces, using cartilage as a model tissue. We chose cartilage for this initial study because it is known to contain tTG[44,45] as well as several ECM substrates of tTG[46–49]. Furthermore, Jurgensen *et al.* previously demonstrated the development of adhesive bonds between cartilage surfaces brought into contact in the presence of tTG, suggesting that protein substrates of tTG are exposed at tissue surfaces and available for reaction with soluble enzyme[50]. Short synthetic peptides containing either lysine or glutamine residues[35] and conjugates of these peptides with polymers were bound to cartilage surfaces through the action of tTG enzyme. Several cartilage ECM components were identified as possible substrates for the reaction, including collagen II, fibronectin, osteopontin and osteonectin. Given the existence of macromolecular tTG substrates in many tissues, this facile approach to tissue surface modification should be broadly applicable to a variety of tissues for localization of therapeutic agents and for enhancing (or inhibiting) adhesion at tissue-tissue and tissue-device interfaces.

Materials and Methods

Materials

Rink amide resin was purchased from Anaspec, San Jose, CA. Fmoc-amino acids were purchased from Peptides International, Louisville, KY. O-(N-Fmoc-3-aminopropyl)-O'-(N-diglycolyl-3-aminopropyl)-diethyleneglycol (MW 558.6) (Fmoc-NH-$(EO)_2$-COOH) was purchased from Novabiochem, San Diego, CA and Fmoc-NH-PEG-NHS ($\bar{M}_w$ = 3.4 kDa) was purchased from Nektar Therapeutics, Inc, Huntsville, AL. Biotin, guinea pig liver tissue transglutaminase (tTG), chondroitinase ABC, fibronectin, and peroxidase conjugated ExtrAvidin were purchased from Sigma, St. Louis, MO. Adult bovine metacarpophalangeal joints were purchased from a local slaughter house. Fluorescein and peroxidase conjugated anti-biotin antibodies and the DAB substrate kit were purchased from Vector Labs,

Burlingame, CA. Anti-Collagen II and Texas Red conjugated donkey anti-rabbit antibodies were purchased from Abcam, Cambridge, MA. Collagen II was purchased from Chondrex, Redmond, WA, osteonectin from Biodesign, Saco, ME, and osteopontin from R&D Systems, Minneapolis, MN. ECL Plus western blotting detection reagents were purchased from GE Healthcare Biosciences, Piscataway, NJ.

Methods

Synthesis of peptide conjugates—The molecules synthesized for use in this study and their abbreviations are listed in Table 1. The peptide portions of the molecules were synthesized on a Rink amide resin (1 g, 0.45 mmol/g) using standard Fmoc solid phase peptide synthesis. Each coupling reaction was performed for 3 hours with a 10 min pre-activation of four equivalents of Fmoc-amino acid: benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP): *N*-hydroxybenzotriazole (HOBt): diisopropylethylamine (DIEA) (1:1:1:1) at room temperature. The Fmoc was removed by 20% piperidine in N-methylpyrrolidinone (NMP) for 1 hour. After Fmoc removal from the final amino acid, either Fmoc-NH-$(EO)_2$-COOH or Fmoc-NH-PEG-NHS was added. Fmoc-NH-$(EO)_2$-COOH was treated as Fmoc-amino acid and used at 2 equivalents. The Fmoc-NH-PEG-NHS was used at 2 equivalents and combined with DIEA (1:2) in dichloromethane (DCM), added to the resin and reacted for 2 days. The unreacted peptide on the resin was acetylated with the use of 4 equivalents of acetic anhydride. The Fmoc was then removed and biotin was coupled using 4 equivalents by the previously described procedure for coupling Fmoc-amino acids. At the completion of the reaction, the resin was washed with NMP, DCM, and methanol (MeOH), two times each. The resin was dried under vacuum and then treated with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane for 3 hours. The crude product was obtained by concentration of the TFA solution and addition of diethyl ether. Purification was performed using semi-preparative RP-HPLC and the masses were confirmed using MALDI-TOF MS analysis on a PE Voyager DE-Pro MALDI-TOF Mass Spectrometer (Perspective Biosystems, MA). The peptide-PEG conjugates were then frozen and lyophilized.

Cartilage isolation and preparation—For the coupling of peptide conjugates to cartilage sections, osteochondral plugs were extracted from adult bovine metacarpophalangeal joints using a hole punch with a diameter of 0.793 cm. Samples were then frozen in tissue freezing media and stored at −80 °C until use. Vertical sections (25 μm), which included the articular surface as well as a portion of the underlying bone, were cut using a cryostat (Microm HM505N, Carl Zeiss) and the freezing media was removed with 3 washes of Tris buffered saline (TBS) (50 mM Tris, 100 mM NaCl) at pH 7.4. For the penetration studies, four millimeter by two millimeter rectangular samples of cartilage were excised from adult bovine metacarpophalangeal joint to a depth of 200–250 microns from the articular surface. Samples were frozen as described earlier for later use, at which time they were thawed and rinsed in TBS.

Coupling of peptide conjugates to cartilage sections—A general scheme of the covalent coupling of the peptide-PEG conjugates to cartilage is shown in Figure 1. Partial depolymerization of glycosaminoglycans[51] in the tissue was accomplished by digesting with chondroitinase ABC at 40 mU/mL in 50 mM Tris, 70 mM sodium acetate and 0.01% bovine serum albumin (BSA) at pH 8 for 30 minutes at 37 °C. Samples were then rinsed 3 times with TBS and blocked with 1% BSA in TBS for one hour at room temperature. The tTG reaction conditions used were similar to a previously described method[45]. The cartilage sections were incubated in 500 μL of 50 μM peptide or peptide-PEG conjugate, 0.5 U/mL tTG, 5 mM $CaCl_2$, 2.5 mM dithiothreitol and 100mM Tris, at pH 8.3 for 30 min at 37 °C. Control experiments were performed in the absence of tTG. After completion of the tTG reaction, the solution was removed and samples were rinsed 3 times with 1% BSA in TBS for 5 minutes

each. The cartilage sections were then incubated with fluorescein conjugated anti-biotin antibody at 10 μg/mL in 1% BSA in TBS for 2 hours at room temperature. Samples were rinsed 4 times with TBS for 5 minutes each, followed by blocking with 10% donkey serum for 1 hour and incubation with rabbit anti-collagen II antibody in TBS containing 10% donkey serum for 1 hour. The samples were rinsed 3 times with 10% donkey serum in TBS and then incubated with Texas Red conjugated donkey anti-rabbit antibody. Samples were rinsed again with TBS and then imaged using a Leica epifluorescent microscope (λ_{ex} = 495 nm, λ_{em} = 520 for fluorescein and λ_{ex} = 596 nm, λ_{em} = 620 for Texas Red) and a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Image analysis—Images were analyzed using Metamorph image analysis software (Molecular Devices, Sunnyvale, CA). Fluorescein fluorescence was measured at three randomly selected areas of the middle zone on each cartilage section image and then averaged. A similar procedure was utilized to determine Texas Red fluorescence. The fluorescein fluorescence intensity was then normalized to the fluorescence of the Texas Red for each cartilage section image in order to account for potential ECM differences between sections. Finally, the average green fluorescence for each peptide-PEG conjugate was then normalized by dividing by the red fluorescence.

Depth of peptide coupling in cartilage—Four millimeter by two millimeter rectangular samples of cartilage were thawed and rinsed, after which the chondroitinase ABC digestion and tTG reactions with the peptide and peptide-PEG conjugates were performed as stated above. (See Figure S1 in supplemental information for schematic of this experimental method.) After the tTG reaction, the cartilage was removed and rinsed 3 times with TBS for 5 minutes each. The samples were then frozen in tissue freezing media followed by cutting using a cryostat. Twenty micron thick vertical sections were collected from each cartilage piece, rinsed in TBS, blocked with 1% BSA TBS containing 0.1% Tween (TBS-T), and incubated with peroxidase conjugated anti-biotin antibody at 5 μg/mL in 1% BSA in TBS-T for 2 hours at room temperature. Samples were rinsed 4 times with TBS and 4 times with distilled water. Finally, the anti-biotin antibody was detected using a DAB kit which contains the peroxidase substrate 3,3′-diaminobenzidine yielding a brown color. Samples were imaged using a Leica microscope (brightfield) and a SPOT RT digital camera.

Adobe Photoshop (Adobe, Inc, San Jose, CA) was used to measure the depth to which the peptide and peptide-PEG conjugates were detected. The depth of staining into the cartilage along the cut surface perpendicular to the articular surface was measured at distances of 60, 120 and 180 microns from the articular surface for each sample. The average depth of staining for each sample was calculated from these three measurements. For each peptide and peptide-PEG conjugate, an overall mean depth of staining was determined from the measurements of three samples.

Reaction of peptides with cartilage ECM proteins—Fibronectin, collagen II, osteonectin, and osteopontin were separately combined at 0.1% (w/v) in 100 mM Tris and 5 mM $CaCl_2$ with 500 μM peptide-PEG conjugates and 0.05 U/mL tTG. The reaction was allowed to proceed for 30 min at 37 °C and then stopped with the addition of iodoacetamide (1 mM final concentration). Control experiments without transglutaminse were also performed. The proteins were then separated using SDS-PAGE with either 7.5% fibronectin and collagen II) or 10% (osteonectin and osteopontin) gels, transferred to PVDF membranes which were then blocked with 2% BSA in TBS with 0.1% Tween (TBS-T). The modified proteins were probed with peroxidase conjugated ExtrAvidin. Detection of bound ExtrAvidin was accomplished using ECL Plus Western Blotting Detection Reagents and imaged on a Storm 860 imager (GE Healthcare Biosciences, Piscataway, NJ). Band intensities were measured using ImageQuant (GE Healthcare Biosciences). Statistical analysis was performed using one-

way ANOVA and Tukey's post-hoc test with 95% confidence intervals with SPSS (SPSS, Chicago, IL). Reactions of B72Q and B72K with osteonectin and osteopontin were dialyzed against 10 mM acetic acid and then analyzed by MALDI-TOF MS.

Results

Fluorescence evaluation of the peptide conjugates coupled to cartilage

In the presence of tTG enzyme, B2K, B2Q, B72K, and B72Q were enzymatically crosslinked to the cartilage sections as evidenced by the images of the tissue sections stained with fluorescein anti-biotin antibody (Figure 2a, d, g, and h). Fluorescence was detected in all regions of the cartilage, from the articular surface to the calcified zone, although fluorescence intensity was greatest near the lacunae. The B2K and B72K treated samples qualitatively appeared to be more fluorescent than the B2Q and B72Q modified cartilage sections.

The catalytic role of tTG in coupling the peptide-PEG conjugates to cartilage was verified with several controls. First, incubation of B2K and B2Q with the cartilage in the absence of tTG resulted in no observed fluorescence (Figure 2b and **e**), indicating that tTG was required for the coupling of the peptide-PEG conjugates to cartilage. Secondly, when cartilage sections were incubated with B2N or B2O in the presence of tTG, no coupling was detected as evidenced by the lack of fluorescence in panels **2c** and **f**. While asparagine and ornithine are structurally similar to glutamine and lysine, respectively, both are known to be poor substrates for tTG due to their shorter side chains.[52–54] Finally, nonspecific binding of the biotin-PEG component of B72K and B72Q to the cartilage can be ruled out as shown by the lack of fluorescence when the cartilage sections were incubated with B72 and tTG (Figure 2i).

Texas Red staining of type II collagen proved to be a useful internal reference for quantifying bound peptide, as red fluorescence was detected in all regions of the cartilage (Figure 3a) and was unaffected by the reaction with peptides and tTG enzyme as can be seen by the level of red fluorescence from the treated samples as compared to that of the untreated samples (Figure 3b). This allowed the amount of peptide bound to the cartilage to be quantified by ratioing green fluorescence from the fluorescein conjugated anti-biotin antibody to that of the red fluorescence resulting from type II collagen staining. Although the average fluorescence intensity from bound peptide was more intense for B2K and B72K samples as compared to B2Q and B72Q samples (Figure 3c), the differences were not statistically significant. The presence of a longer PEG linker did not significantly affect peptide coupling as the fluorescence of B2K and B72K and B2Q and B72Q were similar.

Depth of peptide coupling

In a separate series of experiments, 2 mm by 4 mm by 200–250 μm pieces of cartilage were incubated with each peptide-PEG conjugate and tTG. At the completion of the reaction, the cartilage pieces were frozen and sectioned and the coupled peptide conjugates were detected with a perioxidase anti-biotin antibody. The bound antibody was detected with DAB reagent which forms a brown precipitate in the presence of perioxidase[55]. A representative stained section is shown in Figure 4b. The brown DAB staining was detected on the cut and articular surfaces as indicated by the arrows in Figure 4b. The depth of peptide penetration and coupling was measured along the cut edge at 60, 120, and 180 μm from the articular surface as shown in Figure S1 (supplementary information). No relationship was found between the thickness of DAB staining and distance from the articular surface. The average depth of peptide coupling for B2K was 13 μm. BQ2, B72Q, and B72K were detected at an average depth of 9.8 μm, 8.6 μm, and 9.8 μm, respectively (Figure 4c). Statistical analysis indicated that the observed differences were not statistically significant. In the absence of tTG, no staining was observed (Figure 4a). Finally, a control experiment in which undigested cartilage tissue was treated with

tTG and B2K revealed staining to a depth of only 5.8 microns (data not shown), indicating that digestion with chondroitinase was not required but did enhance the depth of tissue modification.

Reaction of peptide conjugates with cartilage ECM proteins

To identify components of the cartilage ECM that were reactive toward the peptide conjugates, enzyme reactions were conducted in solution with fibronectin, collagen II, osteonectin, and osteopontin. Coupling of peptide conjugates to all proteins was observed as evidenced by western blot analysis (Figure 5a). With the exception of osteopontin, the cartilage proteins reacted more with lysine peptides (B2K and B72K) than with glutamine peptides (B2Q and B72Q) as evidenced by the amount of loaded protein required to detect the biotinylated conjugates. Preferential reactivity of the lysine peptides became evident when the band intensity was normalized by the amount of protein loaded (Figure 5b–e). For fibronectin, type II collagen, and osteonectin the normalized band intensity for B2K and B72K was statistically greater than for B2Q or B72Q ($p<0.05$). As observed with the tissue staining experiments, no differences were found between B2K and B72K and between B2Q and B72Q. For osteopontin, B2Q demonstrated a significantly greater normalized band intensity than the B2K and B72K ($p<0.05$). Control experiments without transglutaminase demonstrated that there were no non-specific interactions between the peptides and the proteins. In addition, reactions of the proteins with B2N, B2O and B72 in the presence of tTG did not demonstrate crosslinking on western blot (data not shown).

The MALDI-TOF spectrum of osteonectin modified with B72K demonstrated that the majority of the protein was modified with one PEG molecule although additional peaks corresponding to protein modified with up to 4 PEGs were detected (Figure S9A). In contrast, when the spectrum of B72Q coupled osteonectin was evaluated, the peak corresponding to unmodified osteonectin was the most intense, indicating that a large portion of the protein had not been modified (Figure S9B). There were also 3 additional peaks visualized, suggesting that osteonectin had been modified with up to 3 molecules of B72Q. The MALDI-TOF mass spectra of osteopontin revealed that majority of this protein was unmodified and that it had reacted with up to two B72Q while only reacting with one B72K (Figure S10).

Discussion

tTG is widely distributed within many connective tissues and has been implicated in organogenesis, tissue repair and in tissue stabilization[41,42]. Cartilage was chosen for our studies in part because a prior study by Jurgensen *et al.* showed that cartilage tissue surfaces adhered strongly to each other when incubated with tTG enzyme, suggesting a high level of reactivity of cartilage ECM components as substrates for tTG[50]. Several components of the cartilage ECM have been identified as substrates for tTG, including osteopontin[56,57], collagen II[45,47], collagen XI[58], fibrillin[59], fibronectin[60], and osteonectin[47,49]. Some other known ECM substrates of tTG found in other tissues include collagen I,[39], [61] collagen V[58], vitronectin[62,63], and laminin[64].

To demonstrate the general approach of tissue surface modification using tTG enzymes, we sought to design a simple and versatile molecular module consisting of a short synthetic peptide substrate that could ultimately be conjugated to a therapeutic molecule such as a peptide, protein, growth factor, drug or synthetic polymer. Specific requirements for tTG reactive glutamine and lysine residues are not fully understood, although it is believed that the enzyme has more stringent requirements for the glutamine (acyl donor) than for the lysine (amine donor) [54]. It has been suggested that a leucine residue flanking the C-terminus of a glutamine increases reactivity[53]. However, recent combinatorial library studies suggest that the amino acid directly adjacent to the reactive glutamine is not as important as the amino acids 2–3 residues away on the C-terminus side[65,66]. Proline and phenylalanine as well as other

nonpolar amino acids have been suggested to have favorable roles in those locations[67,68]. In contrast, tTG is considered more tolerant of lysine substrates, as it has been found to react with a wide variety of primary amines as well as peptide bound lysines[42,54]. Nevertheless, certain peptide features do influence lysine reactivity, such as the presence of a C-terminal adjacent glycine[69] or an N-terminal hydrophobic residue juxtaposed to lysine[35,54]. The specific lysine and glutamine peptides used here were rationally designed and identified as good tTG substrates in our prior study.[35]

Our findings demonstrate that both FKG-NH_2 and GQQQLG-NH_2 are bound to cartilage surfaces via tTG as demonstrated by tissue staining experiments. Conjugates of these peptides with 3.4 kDa PEG were similarly active, suggesting that the presence of the polymer had little effect on the ability of tTG to catalyze the coupling of the peptide to the tissue surface. Diffuse staining over the cartilage matrix as well as more intense focal staining in the pericellular region were observed (Figure 2). The intense staining in the pericellular regions could be explained by tTG reactive noncollagenous proteins which are believed to mediate the interaction between cells and the ECM and can be found predominantly in the areas surrounding the chondrocytes [70]. It is known that some noncollagenous cartilage ECM proteins, such as fibronectin, react well with tTG[41,48,71] and therefore it was anticipated that our peptides would react with the proteins in this region. Intense staining at the articular surface could be due to the high concentration of collagen there[72], although sample geometry and imaging considerations prevent us from making that conclusion without further detailed experiments.

The peptide conjugates were bound to ECM to a depth of approximately 8–13 microns from the cut tissue surface. The depth of tissue modification can be affected by many factors, including the ability of the enzyme and peptide reactants to diffuse into the tissue before reacting with ECM components. No difference was found for the depth of modification when lysine (B2K) or glutamine (B2Q) peptides were coupled to a 3.4 kDa PEG (B72K and B72Q, respectively), suggesting that the diffusion of the peptide components through the tissue is unlikely to be a limiting factor. The masses of all peptide conjugates are well below that of tTG (77 kDa)[73], making it more likely that diffusion of tTG to be a limiting factor. The surface coupling reaction did not require pretreatment of the tissue with chondroitinase, although the depth of tissue modification was greater when the tissue was partially digested. Deeper tissue modification, if desired, may be achieved with a higher concentration of chondroitinase, longer digestion times,[74] or through the use of a different enzyme such as collagenase or hyaluronidase[75].

To correlate the observed tissue modification to specific matrix components of the tissue, we also conducted tTG reactions in solution using selected cartilage ECM proteins and the peptides. One of the major ECM components of cartilage is collagen II, which can be found throughout the cartilage matrix[72]. Collagen II used in these studies was isolated by pepsin digestion, which yields primarily the triple helical region of collagen II[76]. Both lysine peptide conjugates, B2K and B72K, were reactive toward collagen II, suggesting that glutamine residues in the triple helical region act as substrates for tTG. The B2Q and B72Q containing peptides were less reactive toward collagen II in solution, which could be due to loss of lysine residues as a result of hydroxylation during collagen II interchain crosslinking and glycosylation[77].

B2K and B72K were significantly ($p<0.05$) more reactive than B2Q and B72Q with all proteins except osteopontin. There are a few possible explanations for these results. First, FKG has a significantly higher specificity (560 mM^{-1} min^{-1}) than GQQQLG (34.1 mM^{-1} min^{-1})[35], indicating that the amine donor (FKG) is more reactive with the enzyme than the acyl donor (GQQQLG). However, the reactivities of the glutamine and lysine residues in the ECM proteins may also play important roles and could explain the higher reactivity of osteopontin toward

the glutamine peptide conjugates (B2Q and B72Q). In addition, unlike our short peptide constructs that are expected to have no secondary or tertiary structure, the existence of alpha helices, beta sheets and higher order protein structures of proteins may shield some lysine and glutamine residues from the enzyme. For example, it has been demonstrated that when fibronectin is exposed to tTG and [^{14}C]putrescine in solution 4 glutamines are reactive[60]. However, after partial digestion, as many as 8–9 glutamines were reactive toward tTG. Thus, the total number of lysine and glutamine residues in an ECM protein may not always be a reliable indicator of potential reactivity toward tTG.

Conclusion

In summary, our results demonstrate that synthetic peptide and peptide-polymer conjugates can be enzymatically coupled to cartilage under mild conditions through the formation of isopeptide bonds between the peptide and ECM proteins. Cartilage is only one example of a tissue whose free surface is readily accessible through minimally invasive administration of solutions containing tTG and synthetic molecules; others include the tissue surfaces of the oral cavity, gastrointestinal and reproductive tracts, the surface of the eye, and tissue surfaces exposed during invasive surgical procedures. The reactive cartilage ECM components identified in this study can also be found in many other connective tissues, suggesting that this strategy can be broadly applicable. We anticipate that this facile and versatile method for modifying tissue surfaces can be employed with a wide range of therapeutic biomolecules, growth factors and functional polymers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This project was supported by the National Institutes of Health (DE13030 and EB003806) and a National Defense Science and Engineering Graduate Fellowship to MRJ. Dr. James Williams of the Department of Anatomy and Cell Biology, Rush University Medical Center, Rush University, Chicago, IL is acknowledged for his insightful suggestions.

References

1. Slepian MJ, Hubbell JA. Polymeric endoluminal gel paving: hydrogel systems for local barrier creation and site-specific drug delivery. Advanced Drug Delivery Reviews 1997;24:11–30.
2. Elbert D, Hubbell J. Reduction of fibrous adhesion formation by a copolymer possessing an affinity for anionic surfaces. Journal of Biomaterials Research 1998;42:55–65.
3. Winblade N, Schmokel H, Baumann M, Hoffman A, Hubbell J. Sterically blocking adhesion of cells to biological surfaces with a surface-active copolymer containing poly(ethylene glycol) and phenylboronic acid. Journal of Biomedical Materials Research 2002;59:618–631. [PubMed: 11774323]
4. Winblade N, Nikolic I, Hoffman A, Hubbell J. Blocking adhesion to cell and tissue surfaces by the chemisorption of a poly-L-lysine-graft-(poly(ethylene glycol): phenylboronic acid) copolymer. Biomacromolecules 2000;1:523–533. [PubMed: 11710177]
5. Elbert DL, Herbert CB, Hubbell JA. Thin Polymer Layers Formed by Polyelectrolyte Multilayer Techniques on Biological Surfaces. Langmuir 1999;15:5355–5362.
6. Thierry B, Winnik F, Merhi Y, Tabrizian M. Nanocoatings onto arteries via layer-by-layer deposition: Toward the in vivo repair of damaged blood vessels. Journal of the American Chemical Society 2003;125:7494–7495. [PubMed: 12812471]
7. Krol S, del Guerra S, Grupillo M, Diaspro A, Gliozzi A, Marchetti P. Multilayer nanoencapsulation. New approach for immune protection of human pancreatic islets. Nanoletters 2006;6:1933–39.

8. Mercuri J, Lovekamp J, Simionescu D, Vyavahare N. Glycosaminoglycan-targeted fixation for improved bioprosthetic heart vavle stabilization. Biomaterials 2007;28:496–503. [PubMed: 17030363]
9. Lee DY, Yang K, Lee S, Chae SY, Kim KW, Lee MK, et al. Optimization of monomethoxy-polyethylene glycol grafting on the pancreatic islet capsules. Journal of Biomedical Materials Research 2002;62:372–377. [PubMed: 12209922]
10. Zhao C, Sun Y, Amadio P, Tanaka T, Ettema A, An K. Surface treatment of flexor tendon autographs with carbodiimide-derivatized hyaluronic acid. An in vivo canine model. Journal of Bone and Joint Surgery 2006;88:2181–2191. [PubMed: 17015595]
11. Panza J, Wagner W, Rilo H, Rao H, Beckman E, Russell A. Treatment of rat pancreatic islets with reactive PEG. Biomaterials 2000;21:1155–1164. [PubMed: 10817268]
12. Deible C, Beckman E, Russell A, Wagner W. Creating molecular barriers to acute platelet deposition on damaged arteries with reactive polyethylene glycol. Journal of Biomaterials Research 1998;41:251–256.
13. Burchenal J, Deible C, Deglau T, Russell A, Beckman E, Wagner W. Polyethylene glycol diisocyanate decreases platelet deposition after balloon injury of rabbit femoral arteries. Journal of Thrombosis and Thrombolysis 2002;13:27–33. [PubMed: 11994557]
14. Deible CR, Petrosko P, Johnson PC, Beckman EJ, Russell AJ, Wagner WR. Molecular barriers to biomaterial thrombosis by modification of surface proteins with polyethylene glycol. Biomaterials 1999;20:101–9. [PubMed: 10022779]
15. Reyes JMG, Herretes S, Pirouzmanesh A, Wang DA, Elisseeff JH, Jun A, et al. A modified chondroitin sulfate aldehyde adhesive for sealing corneal incisions. Investigative Ophthalmology & Visual Science 2005;46:1247–1250. [PubMed: 15790885]
16. Pirouzmanesh A, Herretes S, Reyes JMG, Suwan-apichon O, Chuck RS, Wang DA, et al. Modified microkeratome-assisted posterior lamellar keratoplasty using a tissue adhesive. Archives of Ophthalmology 2006;124:210–214. [PubMed: 16476891]
17. Ramaswamy S, Wang DA, Fishbein KW, Elisseeff JH, Spencer RG. An analysis of the integration between articular cartilage and nondegradable hydrogel using magnetic resonance imaging. Journal of Biomedical Materials Research Part B-Applied Biomaterials 2006;77B:144–148.
18. Wang D, Varghese S, Sharma B, Strehin I, Fermanian S, Gorham J, et al. Multifunctional chondroitin sulphate for cartilage tissue-biomaterial integration. Nature Materials. 2007In press
19. Wang D, Williams C, Yang F, Elisseeff J. Enhancing the tissue-biomaterial interface: Tissue-initiated integration of biomaterials. Advanced Functional Materials 2004;14:1152–1159.
20. Lorand L, Graham RM. Transglutaminases: Crosslinking enzymes with pleiotropic functions. Nature Reviews Molecular Cell Biology 2003;4:140–156.
21. Yokoyama K, Nio N, Kikuchi Y. Properties and applications of microbial transglutaminase. Applied Microbiology and Biotechnology 2004;64:447–454. [PubMed: 14740191]
22. Chen TH, Janjua R, McDermott MK, Bernstein SL, Steidl SM, Payne GF. Gelatin-based biomimetic tissue adhesive. Potential for retinal reattachment. Journal of Biomedical Materials Research Part B-Applied Biomaterials 2006;77B:416–422.
23. Chen TH, Small DA, McDermott MK, Bentley WE, Payne GF. Enzymatic methods for in situ cell entrapment and cell release. Biomacromolecules 2003;4:1558–1563. [PubMed: 14606880]
24. Schense JC, Bloch J, Aebischer P, Hubbell JA. Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension. Nature Biotechnology 2000;18:415–419.
25. Hall H, Baechi T, Hubbell JA. Molecular properties of fibrin-based matrices for promotion of angiogenesis in vitro. Microvascular Research 2001;62:315–326. [PubMed: 11678634]
26. Ye Q, Zund G, Benedikt P, Jockenhoevel S, Hoerstrup SP, Sakyama S, et al. Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering. European Journal of Cardio-Thoracic Surgery 2000;17:587–591. [PubMed: 10814924]
27. Eyrich D, Brandl F, Appel B, Wiese H, Maier G, Wenzel M, et al. Long-term stable fibrin gels for cartilage engineering. Biomaterials 2007;28:55–65. [PubMed: 16962167]
28. Silverman R, Bonasser L, Passaretti D, Randolph M, Yaremchuk M. Adhesion of tissue-engineered cartilage to native cartilage. Plastic and Reconstructive Surgery 2000;105:1393–1398. [PubMed: 10744230]

29. Peretti GM, Xu JW, Bonassar LJ, Kirchhoff CH, Yaremchuk MJ, Randolph MA. Review of injectable cartilage engineering using fibrin gel in mice and swine models. Tissue Engineering 2006;12:1151–1168. [PubMed: 16771631]
30. Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. Bioconjugate Chemistry 1999;10:75–81. [PubMed: 9893967]
31. Sakiyama SE, Schense JC, Hubbell JA. Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. FASEB Journal 1999;13:2214–2224. [PubMed: 10593869]
32. Schmoekel HG, Weber FE, Schense JC, Gratz KW, Schawalder P, Hubbell JA. Bone repair with a form of BMP-2 engineered for incorporation into fibrin cell ingrowth matrices. Biotechnology and Bioengineering 2005;89:253–262. [PubMed: 15619323]
33. Sanborn TJ, Messersmith PB, Barron AE. In situ crosslinking of a biomimetic peptide-PEG hydrogel via thermally triggered activation of factor XIII. Biomaterials 2002;23:2703–2710. [PubMed: 12059019]
34. Sperinde JJ, Griffith LG. Synthesis and characterization of enzymatically-cross-linked poly(ethylene glycol) hydrogels. Macromolecules 1997;30:5255–5264.
35. Hu B, Messersmith P. Rational design of transglutaminase substrate peptides for rapid enzymatic formation of hydrogels. Journal of the American Chemical Society 2003;125:14298–14299. [PubMed: 14624577]
36. Hu B, Messersmith P. Enzymatically cross-linked hydrogels and their adhesive strength to biosurfaces. Orthodontic and Craniofacial Research 2005;8:145–149.
37. McHale MK, Setton LA, Chilkoti A. Synthesis and in vitro evaluation of enzymatically cross-linked elastin-like polypeptide gels for cartilaginous tissue repair. Tissue Engineering 2005;11:1768–1779. [PubMed: 16411822]
38. Collier JH, Messersmith PB. Enzymatic modification of self-assembled peptide structures with tissue transglutaminase. Bioconjugate Chemistry 2003;14:748–755. [PubMed: 12862427]
39. Chau DYS, Collighan RJ, Verderio EAM, Addy VL, Griffin M. The cellular response to transglutaminase-cross-linked collagen. Biomaterials 2005;26:6518–6529. [PubMed: 15927250]
40. Heath DJ, Christian P, Griffin M. Involvement of tissue transglutaminase in the stabilisation of biomaterial/tissue interfaces important in medical devices. Biomaterials 2002;23:1519–1526. [PubMed: 11829448]
41. Aeschlimann D, Thomazy V. Protein crosslinking in assembly and remodeling of extracellular matrices: the role of transglutaminase. Connective Tissue Research 2000;41:1–27. [PubMed: 10826705]
42. Greenberg CS, Birckbichler PJ, Rice RH. Transglutaminases - Multifunctional cross-linking enzymes that stabilize tissues. FASEB Journal 1991;5:3071–3077. [PubMed: 1683845]
43. Lin CW, Ting AY. Transglutaminase-catalyzed site-specific conjugation of small-molecule probes to proteins in vitro and on the surface of living cells. Journal of the American Chemical Society 2006;128:4542–4543. [PubMed: 16594669]
44. Summey BT, Graff RD, Lai TS, Greenberg CS, Lee GM. Tissue transglutaminase localization and activity regulation in the extracellular matrix of articular cartilage. Journal of Orthopaedic Research 2002;20:76–82. [PubMed: 11853093]
45. Aeschlimann D, Wetterwald A, Fleisch H, Paulsson M. Expression of tissue transglutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes. Journal of Cell Biology 1993;120:1461–1470. [PubMed: 8095503]
46. Aeschlimann D, Mosher D, Paulsson M. Tissue transglutaminase and Factor XIII in cartilage and bone remodeling. Seminars in Thrombosis and Hemostasis 1996;22:437–443. [PubMed: 8989828]
47. Aeschlimann D, Kaupp O, Paulsson M. Transglutaminase-catalyzed matrix crosslinking in differentiating cartilage: identification of osteonectin as a major glutaminyl substrate. Journal of Cell Biology 1995;129:881–892. [PubMed: 7730416]
48. Bowness J, Tarr A. ε(γ-glutaminyl)lysine crosslinks are concentrated in a non-collagenous microfibrillar fraction of cartilage. Biochemistry and Cell Biology 1997;75:89–91. [PubMed: 9192078]

49. Hohenadl C, Mann K, Mayer U, Timpl R, Paulsson M, Aeschlimann D. Two adjacent N-terminal glutamines of BM-40 (osteonectin, SPARC) act as amine acceptor sites in transglutaminase$_c$-catalyzed modification. Journal of Biological Chemistry 1995;270:23415–20. [PubMed: 7559501]
50. Jurgensen K, Aeschlimann D, Cavin V, Genge M, Hunziker E. A new biological glue for cartilage-cartilage interfaces: tissue transglutaminase. Journal of Bone and Joint Surgery 1997;79-A:185–193. [PubMed: 9052538]
51. Hamai A, Hashimoto N, Mochizuki H, Kato F, Makiguchi Y, Horie K, et al. Two distinct chondroitin sulfate ABC lyases. An endoeliminase yielding tetrasaccharides and an exoeliminase preferentially acting on oligosaccharides. Journal of Biological Chemistry 1997;272:9123–30. [PubMed: 9083041]
52. Gross M, Whetzel N, Folk J. Amine binding sites in acyl intermediates of transglutaminase. Journal of Biological Chemistry 1977;252:3752–3759. [PubMed: 16916]
53. Ohtsuka T, Ota M, Nio N, Motoki M. Comparison of substrate specificities of transglutaminases using synthetic peptides as acyl donors. Bioscience, Biotechnolog and Biochemistry 2000;64:2608–2613.
54. Folk J. Mechanism and basis for specificity of transglutaminase-catalyzed ε(γ-glutamyl)lysine bond formation. Advances in Enzymology 1983;54:1–56.
55. Fahmi D, Herzog V. A colorimetric method for measurement of the (peroxidase-mediated) oxidation of 3, 3′-diaminobenzidine. Journal of Histochemistry and Cytochemistry 1973:21. [PubMed: 4736463]
56. Prince C, Dickie D, Krumdieck C. Osteopontin, a substrate for transglutaminase. Biochemical and Biophysical Research Communication 1991;177:1205–1210.
57. Sorensen E, Rasmussen L, Moller L, Jensen P, Hojrup P, Petersen T. Localization of transglutaminase-reactive glutamine residues in bovine osteopontin. Biochemical Journal 1994;304:13–16. [PubMed: 7998923]
58. Kleman JP, Aeschlimann D, Paulsson M, Vanderrest M. Transglutaminase-catalyzed cross-linking of fibrils of collagen V/Xi in A204 rhabdomyosarcoma cells. Biochemistry 1995;34:13768–13775. [PubMed: 7577969]
59. Raghunath M, Cankay R, Kubitscheck U, Fauteck JD, Mayne R, Aeschlimann D, et al. Transglutaminase activity in the eye: Cross-linking in epithelia and connective tissue structures. Investigative Ophthalmology & Visual Science 1999;40:2780–2787. [PubMed: 10549636]
60. Fesus L, Metsis ML, Muszbek L, Koteliansky VE. Transglutaminase-sensitive glutamine residues of human-plasma fibronectin revealed by studying its proteolytic fragments. European Journal of Biochemistry 1986;154:371–374. [PubMed: 2867902]
61. Orban JM, Wilson LB, Kofroth JA, El-Kurdi MS, Maul TM, Vorp DA. Crosslinking of collagen gels by transglutaminase. Journal of Biomedical Materials Research Part A 2004;68A:756–762. [PubMed: 14986330]
62. Sane DC, Moser TL, Parker CJ, Seiffert D, Loskutoff DJ, Greenberg CS. Highly sulfated glycosaminoglycans augment the cross-linking of vitronectin by guinea-pig liver transglutaminase - functional-studies of the cross-linked vitronectin multimers. Journal of Biological Chemistry 1990;265:3543–3548. [PubMed: 1689304]
63. Skorstengaard K, Halkier T, Hojrup P, Mosher D. Sequence location of a putative transglutaminase cross-linking site in human vitronectin. FEBS Letters 1990;262:269–274. [PubMed: 1692291]
64. Aeschlimann D, Paulsson M, Mann K. Identification of Gln(726) in nidogen as the amine acceptor in transglutaminase-catalyzed cross-linking of laminin-nidogen complexes. Journal of Biological Chemistry 1992;267:11316–11321. [PubMed: 1350783]
65. Sugimura Y, Hosono M, Wada F, Yoshimura T, Maki M, Hitomi K. Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library. Journal of Biological Chemistry 2006;281:17699–17706. [PubMed: 16636049]
66. Keresztessy Z, Csosz E, Harsfalvi J, Csomos K, Gray J, Lightowlers R, et al. Phage display selection of efficient glutamine-donor substrate peptides for transglutaminase. Protein Science 2006;15:2466–2480. [PubMed: 17075129]
67. Vader L, de Ru A, van der Wal Y, Kooy Y, Benckuijsen W, Mearin M, et al. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. Journal of Experimental Medicine 2002;195:643–649. [PubMed: 11877487]

68. Fleckenstein B, Molberg O, Qiao S, Schmid D, von der Mulbe F, Elgstoen K, et al. Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Journal of Biological Chemistry 2002;277:34109–34116. [PubMed: 12093810]
69. Groenen P, Smulders R, Peters R, Grootjans J, Van Den Ijssel P, Bloemendal H, et al. The amine-donor substrate specificity of tissue-type transglutaminase. European Journal of Biochemistry 1994;220:795–799. [PubMed: 7908265]
70. Neame P, Tapp H, Azizan A. Noncollagenous, nonproteoglycan macromolecules of cartilage. Cell, Molecular and Life Science 1999;55:1327–1340.
71. Griffin M, Casadio R, Bergamini C. Transglutaminase: Nature's biological glues. Biochemical Journal 2002;368:377–396. [PubMed: 12366374]
72. Goldring, S.; Goldring, M. Biology of the normal joint. In: Harris, E., et al., editors. Kelley's Textbook of Rheumatology. Saunders Co.; St. Louis, MO: 2005. p. 6-26.
73. Ikura K, Nasu T, Yokota H, Tsuchiya Y, Sasaki R, Chiba H. Amino-acid sequence of guinea-pig liver transglutaminase from its cdna sequence. Biochemistry 1988;27:2898–2905. [PubMed: 2900023]
74. Lee M, Sung K, Kurtis M, Akeson W, Sah R. Adhesive forces of chondrocytes to cartilage. Clinical Orthopaedics and Related Research 2000;370:286–294. [PubMed: 10660724]
75. Janssen L, In der Maur C, Bos P, Hardillo J, van Osch G. Short-duration enzymatic treatment promotes integration of a cartilage graft in defect. Annals of Otology, Rhinology, and Laryngology 2006;115:461–8.
76. Rosloniec, E.; Cremer, M.; Kang, A.; Myers, L. Collagen induced arthritis. In: Coligan, J., et al., editors. Current protocols in immunology. John Wiley and Sons, Inc.; Hoboken, NJ: 2007. p. 20-22.
77. Rubin, C.; Rubin, J. Biology, physiology, and morphology of bone. In: Harris, E., et al., editors. Kelley's Textbook of Rheumatology. Saunders Co.; St. Louis, MO: 2005. p. 1449-1469.

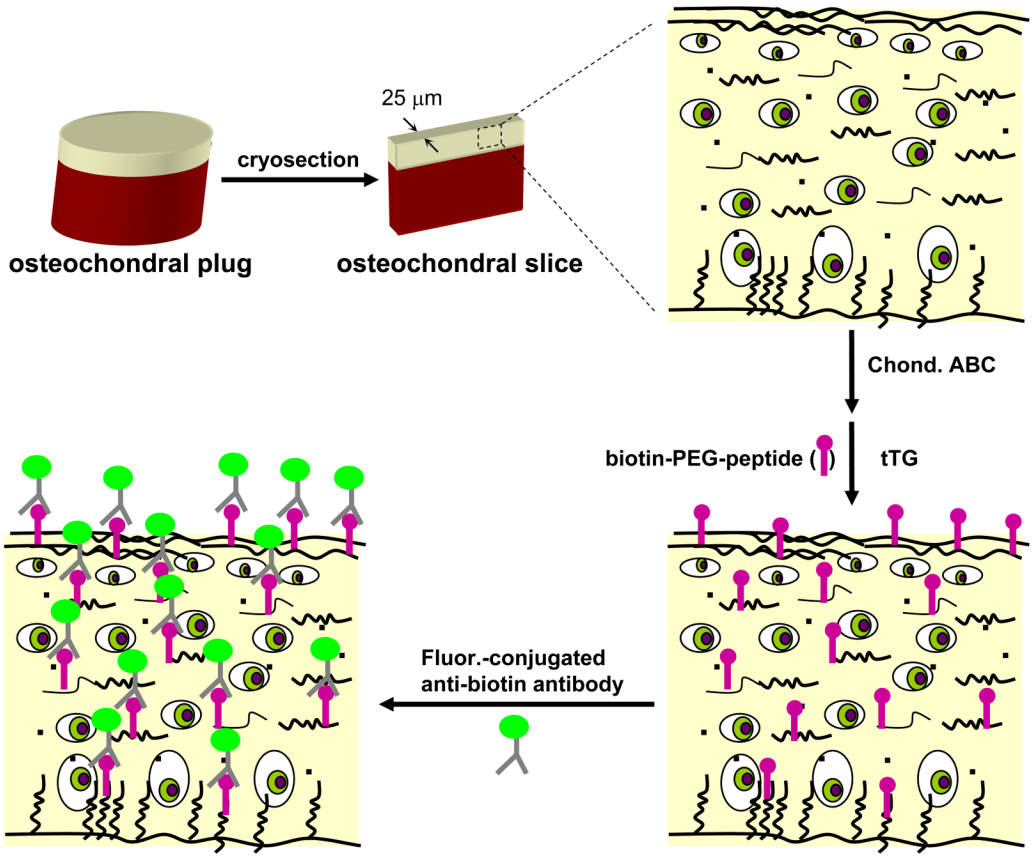

Figure 1.
Schematic overview of the experimental methods used for reaction of synthetic peptides with cartilage tissue surfaces and for detection of tissue-bound peptide-PEG conjugates.

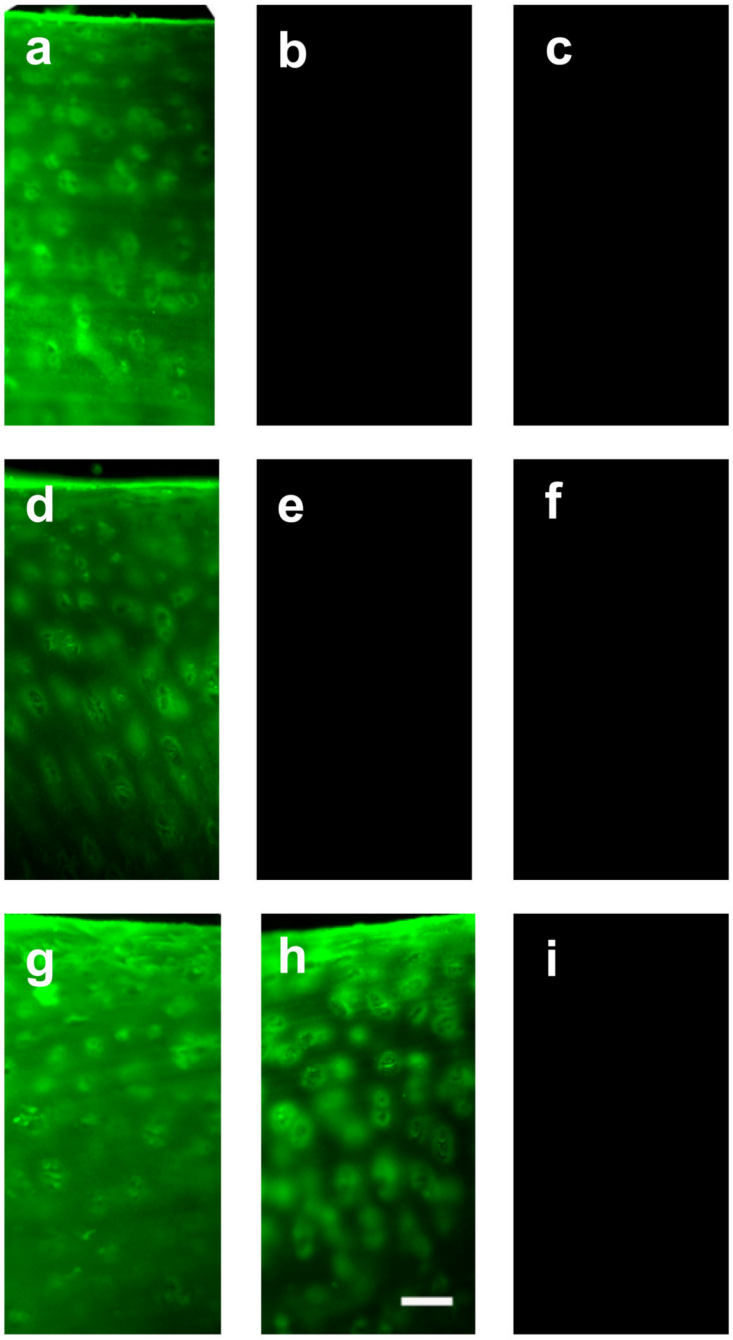

Figure 2.
Representative images of fluorescent anti-biotin antibody stained cartilage sections after reaction with peptide-PEG conjugates and tTG. a) B2K; b) B2K without tTG; c) B2O; d) B2Q; e) B2Q without tTG; f) B2N; g) B72K; h) B72Q; i) B72. Scale bar represents 25 μm for all images. The articular surface is located at the top of each image.

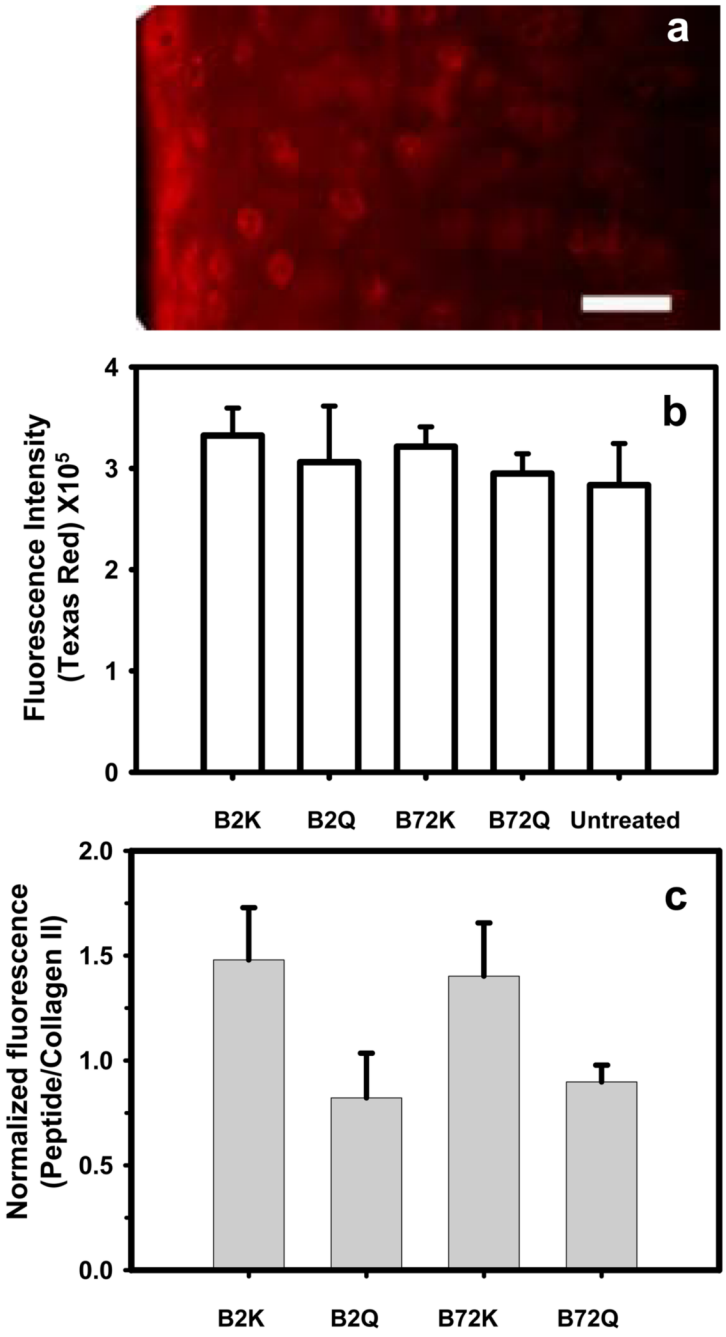

Figure 3.

Quantification of peptide-PEG coupled to cartilage. a) Typical Texas Red fluorescence image of untreated cartilage (the articular surface is shown at the left). Scale bar represents 25 μm. b) Texas Red fluorescence intensity of cartilage sections treated with the peptide-PEG conjugates and tTG. (c) Normalized fluorescein fluorescence intensity of the peptide–PEG treated cartilage. Error bars in b and c represent standard deviation.

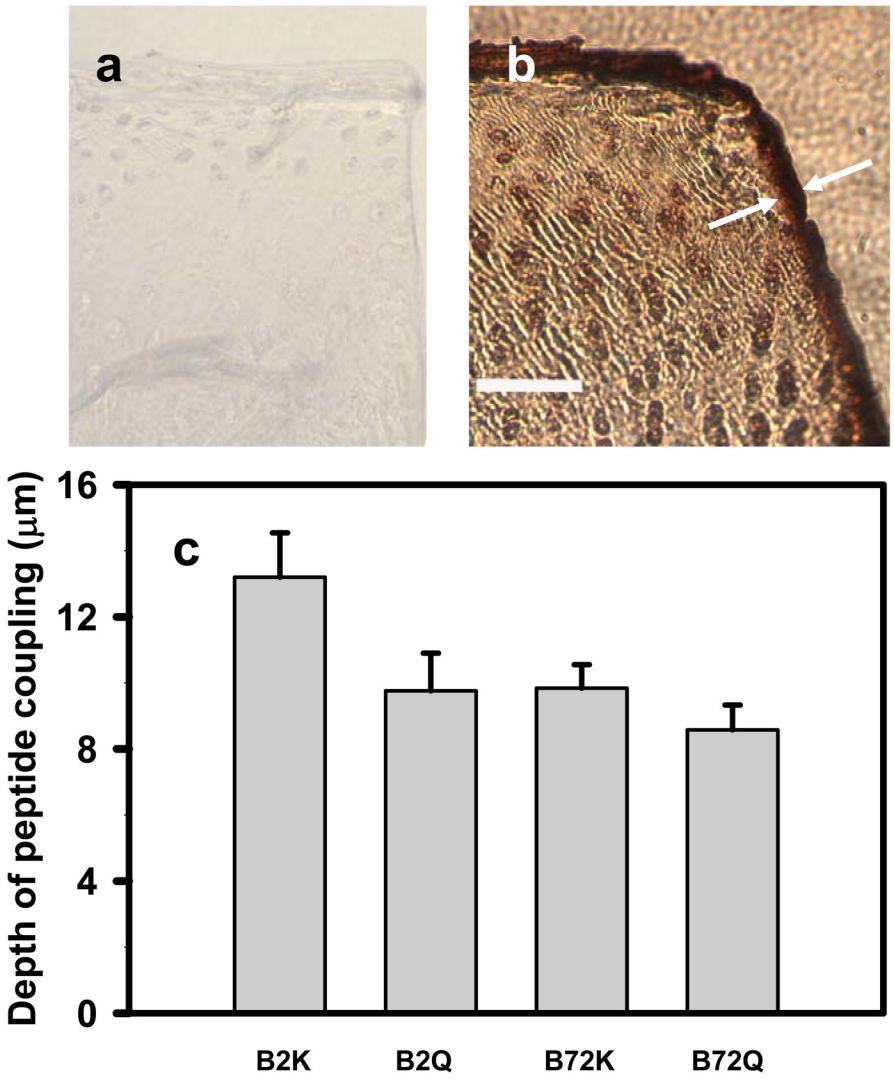

Figure 4.

Depth of peptide-PEG conjugation to cartilage as determined by DAB staining and image analysis. a) Representative image of cartilage treated with B72K peptide but no tTG. b) Representative image of cartilage treated with B2K peptide and tTG. Arrows indicate the region of the cartilage tissue near the exposed surface that has been stained with DAB. c) Graph comparing the thickness of tissue modified by the 4 peptide conjugates. Error bars represent standard deviation. The scale bar in (b) indicates 50 μm for both images.

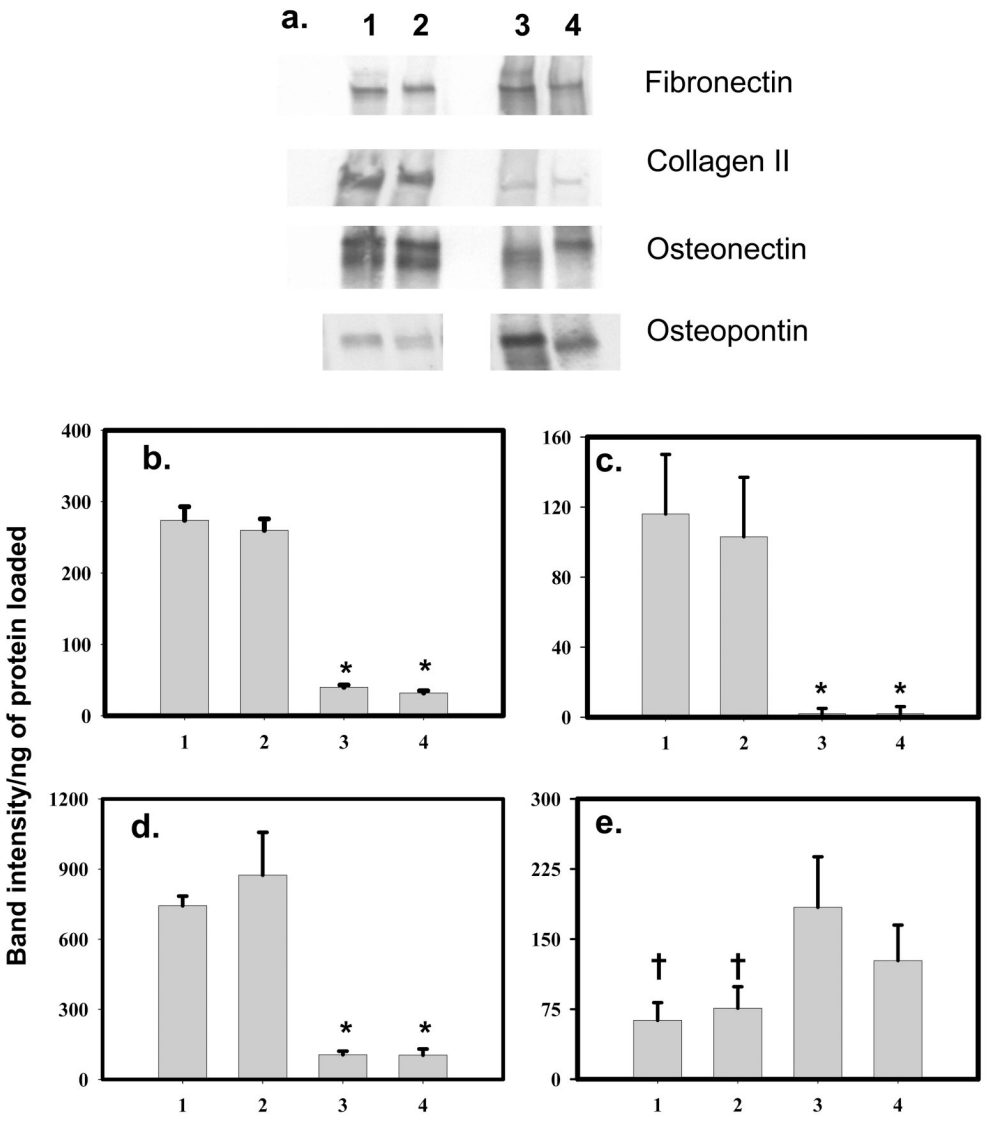

Figure 5.

Reactivity of peptide-PEG conjugates toward cartilage ECM proteins. a) Western blot analysis of individual cartilage proteins, showing their reactivity with each peptide-PEG conjugate. Lane 1 = B2K, Lane 2 = B72K, Lane 3 = B2Q, and Lane 4 = B72Q. b–e. Comparison of the band intensity normalized by the amount of protein loaded. b) Fibronectin. c) Type II collagen. d) Osteonectin. e) Osteopontin. Bar labels are for each peptide-PEG conjugate as defined in panel a. *, $p < 0.05$ as compared to B2K or B72K. †, $p < 0.05$ as compared to B2Q. Error bars represent standard deviation.

Table 1

Structures, abbreviations and mass analyses for synthetic peptide conjugates used in this study.

Structure of peptide-PEG	Abbreviation	Expected Mass (Da)*	Observed Mass (Da)*
Biotin-$(EO)_2$-FKG-NH_2	B2K	894.08	893.49
Biotin-$(EO)_2$-GQQQLG-NH_2	B2Q	1173.34	1172.57
Biotin-$(EO)_2$-FOG-NH_2	B2O	880.05	879.50
Biotin-$(EO)_2$-GNNNLG-NH_2	B2N	1131.26	1131.95
Biotin-PEG-G-NH_2	B72	3600	3600
Biotin-PEG-FKG-NH_2	B72K	3800	3800
Biotin-PEG-GQQQLG-NH_2	B72Q	4100	4100

* values shown for B72, B72K and B72Q are average mass values for polydisperse molecules.