Published in final edited form as:
*Curr Opin Biotechnol*. 2011 October ; 22(5): 655–660. doi:10.1016/j.copbio.2011.01.003.

# Bioconjugation of hydrogels for tissue engineering

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**Keywords**

Tissue; Cell; Pathway Engineering

## Introduction

Tissue engineering is expected to revolutionize regeneration of lost or injured tissues in clinical practice. In this exciting approach, engineered cells along with appropriate growth factors are placed in a supportive matrix and the construct is inserted or injected in the site of regeneration. The implanted construct will guide the organization and commitment of the seeded cells into the desired tissue. There are several products in the market that take advantage of this appraoch to regenerate injured tissues. However, there are still major hurdles to the safe and cost-effective use of engineered constructs in regenerative medicine. The challenge for acellular constructs is homogeneous migration of progenitor cells to the central region of the scaffold. Challenges for cell-based constructs are even greater as the transplanted cells can give rise to undesired lineages due to sub-optimal cell-matrix interactions. In both strategies, cell-matrix interactions play a critical role in guiding the regeneration process. To that end, natural biopolymers have been used extensively as the matrix in tissue engineering [1]. However, there is a need for well-defined tunable matrices with short functional domains to support self-renewal and pluripotency of embryonic and adult stem cells which are cost-effective and easily produced [2]. Hydrogels, due to their high water content and permeability to nutrients, are the material of choice as a replacement for ECM [3*]. Biomolecules can be conjugated to hydrogels to produce a cell-responsive matrix. The discovery that polyethylene glycol (PEG) does not elicit an immune response was a significant milestone, [4**] leading to its wide spread use in tissue engineering as an inert matrix to study cell-biomaterial interaction. The objective of this review is to highlight recent advances in the development of bioconjugated cell-responsive hydrogels and their application in tissue engineering.

## Synthesis and characterization of conjuagted hydrogels

Reaction schemes for conjugation of bioactive peptides to synthetic macromers are presented in Figure 1. Most common coupling reactions include succinimide with amine



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(scheme a), carboxylic acid with amine (scheme b), Michael's addition (scheme c) [5*], and maleimide with thiol (scheme e) [6]. Other conjugation chemistries include iodinate with thiol (scheme d) [7], click reaction of azide with alkyne (scheme f) [8*], and oxime ligation of aldehyde with amine (scheme g) [9*]. Step growth polymerization for simultaneous conjugation of mutiple peptides has been used to synthesize bioconjugated PEG macromers by Michael's addition [10]. In an effort to synthesize injectable hydrogels, tyramine-conjugated succinimide-terminated tetronic acid was crosslinked with hydrogen peroxide and horseradish peroxidase [11*]. The activity of collagenase in cell migration was visualized with a fluorscent dye-modified MMP-degradable peptide conjugated PEG hydrogel [12].

## Conjugated hydrogles in tissue engineering

Synthesis of bioconjugated hydrogels goes back to 1990s when triblock artificial proteins consisting of leucine zipper domains attached to a PEG domain were shown to self-assemble and undergo reversible gelation [13**]. More recently, triblock maleimide-thiol conjugated peptide-PEG-peptide macromers, inspired by the coiled-coil domain of fibrin, were self-assembled into a hydrogel with negligible effect on the peptide secondary structure [14]. Hybrid gels with functional protein domains containing secondary α-helical or β-sheet structures have also been explored [15]. The concept of a multi-block artifical protein network was later extended to the synthesis of peptide-conjugated hydrogels for applications in tissue engineering in the late 1990s. To recapitulate the process of matrix degradation by migrating cells, acrylate-terminated triblocks of PEG and matrix metalloproteinase- (MMP) degradable or plasmin-degradable peptides were crosslinked to produce enzymatically-degradable hydrogels [16**]. Cells infiltrated the conjugated PEG hydrogel after implantation in calvaria [17]. A functionalized poly(ethylene oxide-co-lactide) macromer was crosslinked with MMP-degradable crosslinker to form hydrolytically as well as enzymatically degrading hydrogels for cell encapsulation [18]. The above works demonstrated that short peptides bound to ECM proteins are biologically active when conjugated to an inert hydrogel.

Cell adhesion is mediated by the interaction of surface receptors with ligands bound to ECM proteins. For mimicking that process, PEG hydrogels conjugated with integrin-binding RGD peptide dramatically increased adhesion of fibroblasts [19**] and endothelial cells [20]. Encapsulation of stromal cells in RGD-conjugated hydrogels substantially increased osteogenesis and mineralization [21]. It is important to note that the extent of cell adhesion to RGD-conjugated PEG hydrogels was significantly less than those on collagen-coated substrates signifying that other factors such as substrate structure and RGD presentation as well as other ECM bound ligands are involved in cell adesion. Chondrocytes encapsulated in RGD-conjugated alginate gels enhanced glycosaminoglycans production compared to untreated gels [22]. PEG hydrogels with controlled RGD spatial organization improved adhesion of endothelial cells [23]. The amino acid sequence -$(Pro–Hyp–Gly)_x$- (CMP) forms a triple helix conformation that associates with collagen-I. PEG hydrogels conjugated with tyrosine-terminated CMP increased glycosaminoglycan and collagen content of encapsulated chondrocytes [24]. A non-adhesive PHSRN sequence in the $9^{th}$ type III repeating unit of fibronectin modulates cell adhesion activity of RGD domains in the $10^{th}$ repeating unit. PEG hyrogels were conjugated with RGD peptide and its synergistic PHSRN site linked via an oligoglycine sequence, to recapitulate their native spacing. Adhesion and metabolic activity of neonatal osteoblasts on the conjugated hydrogels were significantly higher than those with RGD alone [25]. Osteoblasts seeded in hydrogels conjugated with RGD and sialoprotein-bound FHRRIKA peptide had significantly higher proliferation rate compared with those with RGD or FHRRIKA alone [26]. The above works illustrate that synergistic activity of mutiple active domains in a peptide is maintained after conjugation to

an inert hydrogel. It is interesting to note that *in vivo* cell infiltration in PEG hydrogels depended on both RGD density and MMP enzymatic activity [27]. In an effort to improve osteogenic differentiation and mineralization of bone marrow stromal cells (MSCs), orthogonal conjugation was used to covalently attach cell-adhesive RGD and osteoinductive BMP2 peptides to a hydrogel [28*]. RGD and BMP2 conjugation synergistically enhanced osteogenic differentiation and mineralization of MSCs. These works demonstrate the need for developing synthetic methods to conjugate several biomolecules to the scaffold for controlling multiple cell functions. Figure 2 illustrates schematically three different peptides conjugated orthogonally with different patterns to a hydrogel substrate.

Polysaccharides such as hyaluronic acid (HA) and heparin are known to bind reversibly to many proteins to selectively activate the desired cell functions. Peptide-conjugated HA-PEG hydrogels showed hyaluronidase-dependent degradation and improved cell viability [29]. To improve survival and diffferentiation of neural stem cells, amine-terminated PEG was reacted with heprin and conjugatd with RGD peptide to form a hydorgel with controlled matrix elasticity, cell adhesion and survival [30*]. Soluble mitogens like FGF-2, immoblized in the hydrogel by non-covalent interaction with heparin, promoted adhesion and differentiation of neural stem cells. In an effort to increase insulin secretion from islet cells, glucagon-like peptide-1 (GLP-1) was conjugated to a water-soluble polymer and encapsulated with the islets [31]. Conjugation prolonged GLP-1 bioactivity and signifcantly increased insulin secretion. However, the activity of conjugated GLP-1 was less than that of native peptide, apparently due to participation of GLP-1 histidine residue in the coupling reaction. Therefore, conjugation reactions should be carfeully designed to minimize interference from coupling moieties with ligand-receptor interactions. PEG hydrogels conjugated with RGD and an inhibitory peptide from interleukin-R1 protein protected the encapsulated β-cells against low molecular weight cytotoxic compounds secereted by T-lymphocytes [32]. Similarly, adrenal pheochromocytoma cells, pancreatic islets, or hMSCs encapsulated in PEG hydrogels conjugated with a peptide sequestering the pro-inflammatory cytokine TNFα were unaffacted by the infiltrated TNFα [33]. PEG hydrogels conjugated with apoptosis inducing anti-Fas monoclonal antibodies provided localized protection for encapsualated islet cells. The hydrogel induced apoptosis specific to FAS-sensitive T-cells, demonstrating retention of antibody specificity after conjugation to the hydrogel [34*]. To stabilize myocardial infarct, VEGF conjugated to a temperature-sensitive copolymer and injected around the infarct, produced higher blood vessel density and improvement in venticular function compared with VEGF mixed with the hydrogel [35]. Implantation of a chitosan construct conjugated with an angiopoietin-1 bound peptide reduced cell apoptosis and promoted cardiomyocyte cell elongation [36].

Enzymes in the native ECM have been used to bond conjugated hydrogels to cartilage. In this approach, lysine- and glutamine-conjugated PEG was covalently attached to cartilage in the presence of transglutaminase enzyme that catalyzes the reactions of lysine/glutamine residues [37]. Conjugation has also been used to improve mechanical properties of composite hydrogels using an osteonectin-bound glutamic acid sequence (Glu6). When a functionalized macomer was crosslinked in the presence of acrylated-Glu6-treated apatite crystals, there was a dramatic increase in the hydrogel modulus which was modulated by the size of the apatite crystals [38*]. RGD conjugation above a critical density to alginate gels not only promoted spreading of encapsulated myoblasts but also provided additional mechanical integrity via calcium-mediated crosslinking [39]. Recently, a PEG hydrogel has been developed for remote manipulation of gel properties and dynamic tuning of local microenvironment via a photolabile RGD network [40**]. Localized removel of photolabile RGDS, connected to a photolabile nitrobenzyl ether moiety susceptible to photolysis, increased chondrogenic differentiation and expression of encapsulated human MSCs. These

hydrogels allow localized real-time manipulation of matrix properties to study the effect of microenvironment on cell function.

Human embryonic stem cells (hESCs), due to their capacity for self-renewal and differentiation into multiple lineages are an attractive cell source for creating 3D functional tissues. However, controlling their differentiation into a specific lineage poses a major challenge. Toward that end, MSCs derived from hESCs seeded in RGD-conjugated PEG hydrogels restored the normal organization and architecture of cartilage after implantation [41]. Peptide conjugated hydrogels have also been developed for maintaining pluripotency and self-renewal of hESCs. In this approach, acrylate-coated substrates were conjugated with integrin-binding peptides from the active domains of ECM proteins [42**]. hESCs cultured on the modified surfaces maintained their capacity for self-renewal with morphology and phenotype similar to those on matrigel. The above works illustrate the vital role of conjugated hydrogels as a carrier for delivery and guiding differentiation of hESCs. DNA conjugated hydrogels have been developed to produce functional proteins without living cells. In this apprach, a solution containing crosslinkable DNA, plasmid gene of interest, and DNA ligase was crosslinked into precisely defined micropads [43**]. Protein expression was observed in the micropads when incubated with the appropriate cell lysate. This approach could potentially be used to guide cell function and ECM production *in situ* in tissue engineered scaffolds.

Directional cell migration, in response to a ligand gradient, plays a key role in tissue morphogenesis as well as guiding the migration of host cells. Smooth muscle cells preferentially migrated toward higher densities on bFGF-gradient hydrogels compared to those without a gradient [44]. In another study, a gradient in RGD density was formed within a microchannel in agarose gel modified with photolabile nitrobenzyl cysteine by laser micropatterning [45**]. Dorsal root ganglia cells migrated and grew within the RGD-conjugated microchannel and not in the surrounding untreated agarose gel. The above technique allows immobilization of biomolecules in selected volumes of a 3D hydrogel matrix for guided tissue regeneration. Parallel clusters of chondrocytes with precise size and shape, within a hydrogel, regulated spatial organization of ECM via cell-cell interaction [46]. Photolithography, microfluidics, and micromolding are very useful for engineering the microscale structure in hydrogels [47*]. When cells with innate ability to align were seeded in 3D micropatterened gelatin hydrogels, they aligned with neighboring cells and self-organized into functional tissues but the extent of alignment depended on the size of micropatterns [48]. Organ printing applies layered deposition of cells from a hydrogel solution to create complex 3D structures with anatomical cell and growth factor arrangements [49**]. Natural biopolymers have been used extensively as a matrix in 3D printing. However, organ printing with synthetic hydrogels conjugated with biomolecules is less common [50]. There is a need to merge conjugation chemistry, hydrogel synthesis, and 3D printing techniques to create well-defined tunable structures with anatomical arrangement of cells and biomolecules (see Figure 2).

## Conclusions/future outlook

A variety of biomolecules including short peptides, proteins, polysaccharides, antibodies, and DNA molecules have been conjugated to hydrogels for controlling cell survival, adhesion, matrix degradation and organization, migration, differention, and apoptosis. The results of these studies clearly demonstrate that biomolecules maintain their activity after conjugation to an othewise inert hydrogel networtk. Future research should address the following issues:

1. Investigate synergistic effect of conjugating several biomolecules to a hydrogel on cell function

2. Merge micro- and nanopatterning techniques with conjugation chemistry to investigate cell function on patterned hydrogel substrates
3. Develop novel hydrogels conjugated with biomolecules that support self-renewal and pluripotency of embryonic and adult stem cells
4. Merge organ printing with bioconjugated hydrogels to fabricate well-defined tunabl structures with anatomical arrangement of cells and biomolecules
5. Evaluate bioconjugated hydrogels *in vivo* in animal models and human clinical trials.

## Acknowledgments

Funding was provided by NSF (CBET0756394 & CBET0931998), NIH (DE19180), and AO Foundation (C10-44J). E. Jabbari thanks Dr. Xuezhong He for preparation of the manuscript.

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** of outstanding interest

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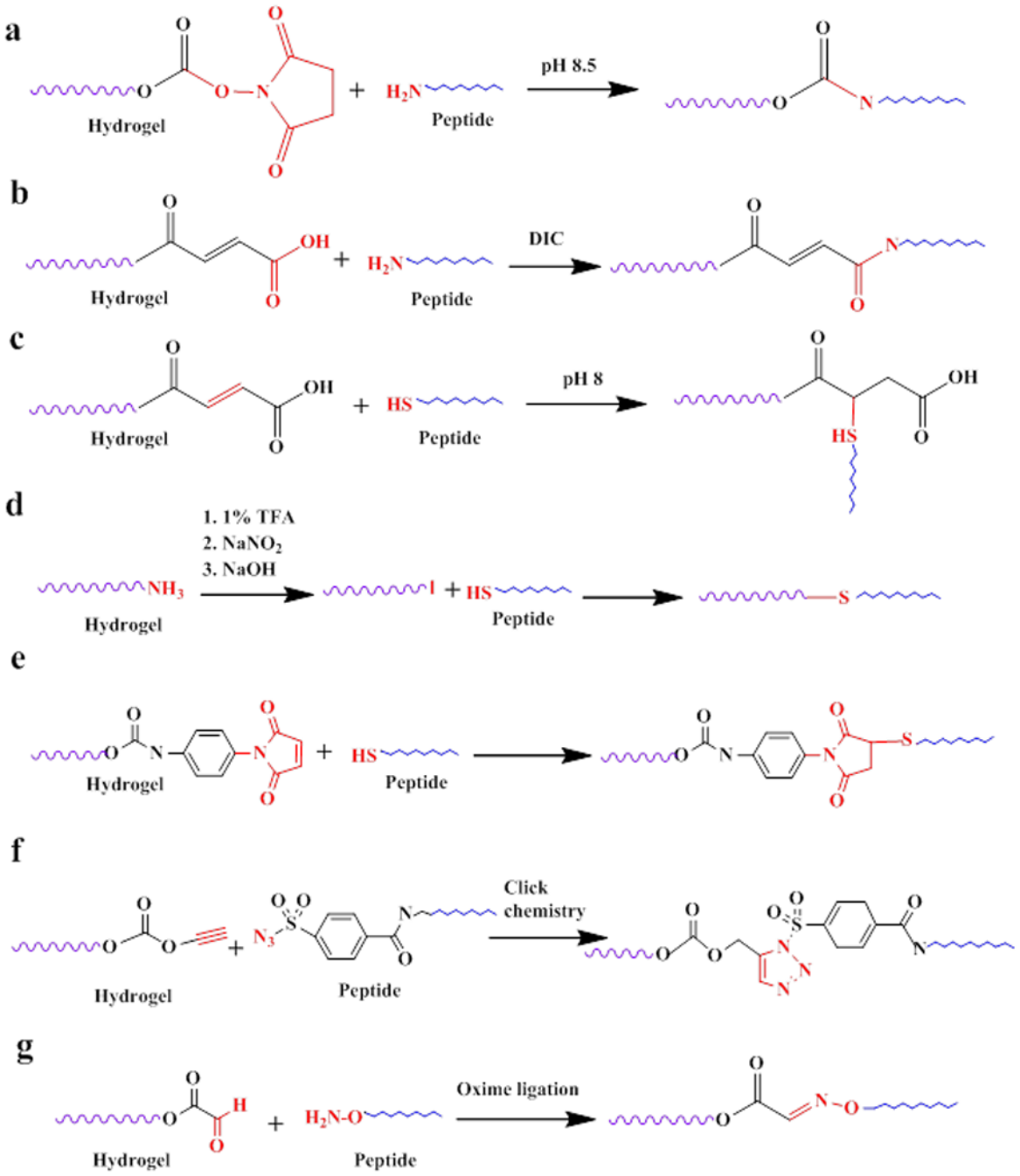


**Figure 1.**
Reaction schemes for bioconjugation.

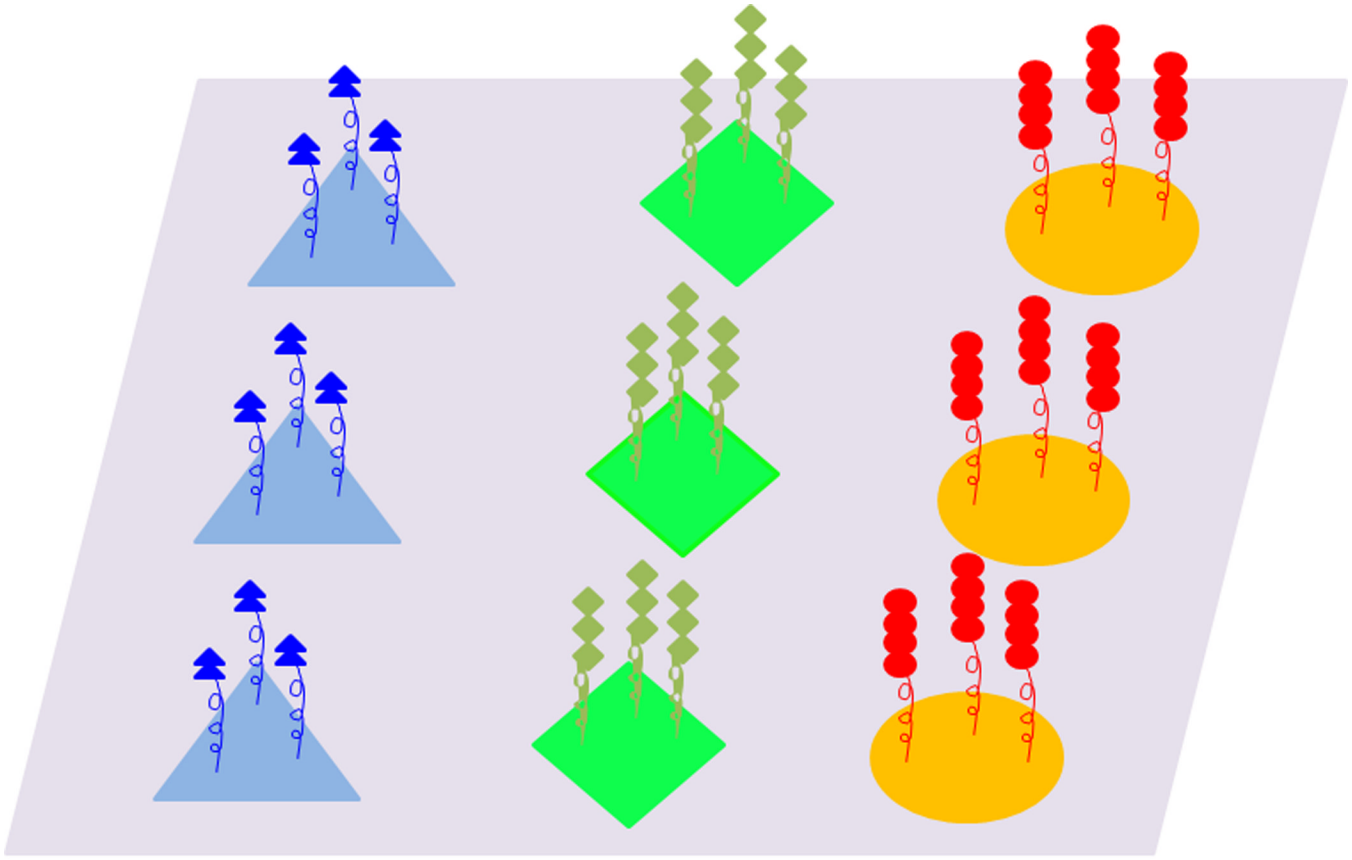

**Figure 2.**
Schematic diagram illustrating three different biomolecules (blue, green and red) conjugated in different patterns (triangle, diamond, and elipse) to a hydrogel substrate. A biomolecule is a short peptide, protein, polysaccharide, antibody, or DNA molecule. The patterns are intended to mimic the spatial organization of biomolecules in the desired native tissue.