50c Solid-Phase Synthesis of Functionalized Peptides as Enzymatically Degradable Crosslinkers for Fabrication of Tissue Engineering Scaffolds

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Biomaterials seeded with cells and coupled with minimally invasive techniques are an attractive alternative for treating irregularly shaped defects with minimum tissue dissection and retraction. After hardening in-situ, these three dimensional matrices can guide the organization, differentiation, proliferation, and development of seeded cells into the desired tissue. Biodegradable scaffolds provide the opportunity to form a completely new tissue that can adapt to natural tissue functions as the matrix degrades. Degradable scaffolds fabricated from polymers such as poly (lactic-co-glycolic acid) that degrades by hydrolysis cannot provide the degradation characteristics required for tissue remodeling. The ability to degrade extracellular proteins is essential for any individual cell to interact properly with its immediate surroundings and for multicellular organisms to develop and function normally.

The matrix metaloproteinases (MMPs) constitute a family of secreted and cell surface enzymes that degrade numerous structural extracellular matrix proteins such as collagen. It is well established that MMPs act on and degrade specific sequences of amino acids on the collagen fibrils. For example, osteoblasts secret MMP-13 that cleave the amino acid sequence Pro-Gln-Gly-Leu-Ala-NH2 of human type II collagen. This peptide sequence has been synthesized by solid-phase peptide synthesis methodology and functionalized with acrylate end-groups in solution for use as a crosslinker in fabrication of biologically degradable hydrogel scaffolds. Normally, these peptide sequences, after synthesis and cleavage from the solid support, are functionalized in solution requiring extensive purification by dialysis. In this report, we present a novel method for synthesis of functionalized peptide cross-linkers.

Rink amide resin, HoBt and Fmoc-protected amino acids were purchased from Novabiochem (San Diego, CA). Peptide synthesis grade reagents 4-Dimethylaminopyridine (DMAP), 1,3-diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), dichloromethane (DCM), dimethyl formamide (DMF), and acetonitrile (MeCN) were purchased from Acros. All reagents were used as received without any further purification. RGD peptide and MMP-3 degradable peptide with unsaturated reactive end groups were synthesized manually using a novel method on the Rink Amide NovaGelTM resin (NovaGel^{TM}, 0.62 mmol/g). Resin (100 mg) was swelled in DMF (3 ml) for 1h and washed with DMF (3 ml). The Fmoc-protected amino acid derivative (6 eq), HOBt (12 eq), and DIC (6.6 eq) in dry DMF (3 ml) were mixed and added to the resin. 0.2 ml of 0.05 M DMAP was added to this mixture and agitated with an orbital shaker for 4-6 h at 30°C. A small volume of the resin was removed and tested for the presence of unreacted amine groups using Kaiser reagents. If the test result was positive, the resin was washed with 3 ml of DMF and the coupling reaction was repeated until a negative result was obtained. Then, the resin was treated with 20% piperidine in DMF for 15 min, washed with DMF, and other amino acids were coupled successively using the same procedure. After coupling the last amino acid of the peptide chain, the resin was washed with 5ml of DMF and DCM. The Mtt protecting group was selectively deprotected by treating the peptidyl resin with 3 ml of TFA/DCM (1:99 v/v) for 2 min. The mixture was filtered and treated with the same volume and ratio of TFA/DCM 7 times. The resin was washed with 3x3 ml of DCM and DMF and the Fmoc protecting group was removed by treatment with 20% piperidine in DMF for 2x15 min. The resin was washed thoroughly with 5x3ml of DMF.

Bifunctional peptide with unsaturated acrylate end groups was synthesized on resin by coupling acrylic acid to the amine groups of glutamine and lysine residues at the two ends of the peptide sequence.

Briefly, acrylic acid (12 eq), HoBt (24 eq), and DIC (13.2 eq) were mixed in 3 ml of DMF, added to the peptidyl resin, and mixed in an incubator shaker at 30°C for 6 h. The Kaiser reagents were used to test for the completion of the coupling reaction. If the test result was positive the coupling reaction was repeated until a negative test result was obtained. The resin was washed with 3x3 ml of DMF and DCM and treated with a mixture of 95% TFA, 2.5% TIPS, and 2.5% water for 2 h to cleave the peptide from the resin and deprotect the side chains. The combined cleavage solution was added to cold ether at -20°C for 24 h to precipitate the crude peptide cross-linker. The suspension was centrifuged, and the supernatant was decanted. The pellet was dried for 2 h under vacuum. The crude product was purified by preparative HPLC on a 250x10 mm, 10 mm Xterra® Prep RP18 column (Waters, Milford, MA) at a flow rate 2 ml/min using a gradient 5% MeCN and 95% 0.1% aqueous TFA solvent mixture. A photodiode array detector (model 996, Waters) was used for detection at a wavelength of 214 nm. The fractionated and purified peptide was freeze-dried and stored at -80°C until used.

Hydrogels as a cell carrier were prepared using a novel poly (lactide-ethylene oxide-fumarate) terpolymer as the macromer, peptide crosslinker degradable by MMP-13, and a neutral redox initiation system. The redox system consisted of ammonium persulfate (APS) and tetramethylethylenediamine (TMEDA), respectively. In a typical procedure, 0.63g of PLEOF and 0.3 g of crosslinker were added to 1.65 ml of PBS and vortexed. To this mixture, 0.21 ml of 0.3 M APS (Aldrich) and 0.21 ml of 0.3 M TMEDA (Aldrich) were added and vortexed. The mixture was degassed, injected between two glass plates separated by a 0.5 mm gap, and fastened with clips. The assembly was placed in a convection oven at 37°C for 15 min to crosslink. The concentrations of APS and TMEDA in the final solution were each 0.03 M. After crosslinking, the gel was removed from the glass plate and disks were cut from the gel using a 15 mm cork-borer. The disk-shaped samples were used for swelling, cell viability, cell function, and degradation studies. The results of swelling studies, cell adhesion, and degradation by collagenase will be presented.