Polymeric Biomaterials with Degradation Sites for Proteases Involved in Cell Migration

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A new class of biodegradable polymeric biomaterials is described which exhibits degradation by specific enzymes that are localized near cell surfaces during cell migration. These materials are telechelic BAB block copolymers of the water-soluble biocompatible polymer poly(ethylene glycol) (the A block, abbreviated PEG hereafter) and short oligopeptides that are cleavage sequences for targeted enzymes (the B block). These copolymers are further capped at each end with reactive acrylate groups to allow polymerization to form cross-linked hydrogel networks. Two materials were synthesized, one specifically degraded by collagenase and the other by plasmin. The incorporation of peptides that induce cell adhesion to similar materials has been previously reported.[1] The combination of these two types of bioactive signals (one for cell-mediated degradation, one for cell adhesion) into one material should achieve a biomimetic polymeric hydrogel with properties of natural extracellular matrix (also a polymeric hydrogel, although of biological origin), while still affording the advantages of a synthetic polymer. These materials are expected to have numerous applications in wound healing and tissue engineering.

During processes such as wound healing and angiogenesis, migrating cells secrete or locally activate a number of enzymes to degrade and remodel matrix macromolecules in their pathway.[2−4] Matrix metalloproteases (MMPs) and plasmin have been identified as particularly important proteases for cell migration, as described below.

Collagen is an important structural protein in the extracellular matrix of most tissues, and its breakdown during cell migration is mediated by the MMP family. A prominent member of this protease family is MMP I or fibroblast collagenase, simply referred to as collagenase hereafter.[5−10] Migrating cells manipulate the balance of collagenase and inhibitors of collagenase to maintain a proteolytic environment that is localized near the cell but is inhibited farther away from the cell surface.[11−13] A variety of signals, both immobilized and diffusible, have been demonstrated to modulate this balance in remodeling processes such as wound healing.[3,14−17]

The sensitivity of collagen to collagenase has been extensively investigated.[18] In human type I collagen, a form of collagen most commonly involved in load-bearing tissues, a specific site (Gly_{775}-Ile_{776}) is cut in the α1(I) chains of the triple-helical collagen.[18−20] While cleavage in the intact collagen chain is quite specific, cleavage in smaller peptides is much less specific. In most naturally occurring substrates, a Gly residue is present in the P1 position (i.e., immediately N-terminal to the cleaved peptide bond, using the notation of Schechter and Berger[21]), and a bulky hydrophobic amino acid residue such as Ile or Leu is found at the P1′ position (i.e., immediately C-terminal to the cleaved peptide bond).[19,22] The sequence Ala-Pro-Gly-Leu was selected as an initial candidate collagenase substrate for these studies, where cleavage was expected between the Gly and Leu residues.

Plasmin is a second enzyme that plays a key role in cell migration, especially during wound healing.[23,24] Tissue injury is immediately followed by coagulation (i.e., thrombin-mediated polymerization of fibrinogen) and the formation of a fibrin-containing provisional matrix; this matrix is replaced with viable tissue during wound healing.[11,25] For cells to invade and remodel this provisional matrix, they must be able to degrade it with fibrinolytic enzymes; plasmin is a fibrinolytic protease that is generated by cleavage of the precursor plasminogen by urokinase plasminogen activator or tissue plasminogen activator. The activity of plasmin appears to be locally regulated, in this case via receptors on the cell surface that bind plasminogen activators and thereby locally activate the fibrinolytic enzyme.[4,12,24,25] In an in vitro model of peripheral nerve regeneration through fibrin matrices, for example, an intact fibrin structure was observed within 100 nm of the surface of the extending nerve growth cones.[26] As is the case with collagenase, a number of biological signals associated with cell adhesion, migration, and injury have been observed to regulate local plasmin activity.[27,28]

The sensitivity of fibrin to cleavage by plasmin has been extensively characterized. Plasmin is able to cleave fibrinogen (the precursor of fibrin) at several locations, including at Arg_{104}-Asp_{105}, Arg_{110}-Val_{111}, and Lys_{206}-Met_{207} on the α-chain; at Arg_{42}-Ala_{43} and Lys_{130}-Glu_{131} on the β-chain; and at Lys_{84}-Ser_{85} and Lys_{87}-Met_{88} on the γ-chain.[29] The sequence Val-Arg-Asn was selected as an initial candidate plasmin substrate for these studies, based on the role of the Arg residue in plasmin sensitivity, where cleavage was expected between the Arg and Asn residues.

Methods. Poly(ethylene glycol) (MW 6000, Fluka) was dehydrated by azeotropic distillation of water in toluene, precipitated with cold ethyl ether, filtered, washed with ether, and dried in vacuo. To synthesize a collagenase-sensitive polymer, the peptide Ala-Pro-Gly-Leu was utilized as a copolymer block. A 16 mmol sample of amine-protected leucine (Fmoc-Leu-OH) was dissolved in a mixture of 80% dichloromethane (DCM) and 20% dimethylformamide (DMF) under argon. All solvents were anhydrous. An 8 mmol sample of dicyclohexylcarbodiimide (DCC) was added and allowed to react for 30 min at room temperature under argon. The urea precipitate resulting from reaction was removed by filtration, and the filtrate was added to 6 g (ca. 1 mmol, or 2 mmol of PEG-OH end groups) of dry PEG in DCM with 1% anhydrous pyridine. The mixture was

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allowed to react for 6 h at room temperature under argon, forming an ester link between the PEG chain terminus and the first amino acid, C-terminal in the sequence of the peptide block.[30] The polymer was precipitated with cold ether, filtered, washed with ether, and dried in vacuo. The Fmoc protecting group was removed with 20% piperidine in DMF, and the polymer was precipitated, filtered, washed, and dried in vacuo. The above steps were repeated for Fmoc-Gly-OH, Fmoc-Pro-OH, and Fmoc-Ala-OH. At each step, the addition of the amino acid was confirmed by measuring the absorbance of the Fmoc protecting group at 275 nm and, after deprotection, quantifying amine groups with the ninhydrin assay. The resulting material is denoted NH_2−(Ala-Pro-Gly-Leu)−PEG−(Ala-Pro-Gly-Leu)−NH_2. (The $-NH_2$ indicates the N-terminus of the peptide; the peptide sequences are written according to usual standards from the N-terminus to the C-terminus; the N-termini (the two Ala residues) are free, and the C-termini (the two Leu residues) are conjugated to the PEG chain.) This material was dissolved in DCM with equimolar triethylamine, and a 2-fold molar excess of acryloyl chloride was added dropwise. This reaction was allowed to proceed for 4 h at room temperature under argon, yielding H_2C=CH−C(=O)−NH−(Ala-Pro-Gly-Leu)−PEG−(Ala-Pro-Gly-Leu)−NH−C(=O)−CH=CH_2 (abbreviated throughout the remainder of this paper as Acr-(APGL)-PEG-(APGL)-Acr, using the single-letter amino acid nomenclature). Proton NMR was used to quantify the degree of acrylation, and the disappearance of amine groups was assessed using the ninhydrin assay to judge the efficiency of coupling of each successive amino acids residue. Gel permeation chromatography with a photodiode array detector and an evaporative mass detector was used to determine the molecular weight of the product. A plasmin-sensitive polymer was synthesized using the same methodology but with the amino acid sequence Val-Arg-Asn, so as to yield the copolymer H_2C=CH−C(=O)−NH−(Val-Arg-Asn)−PEG−(Val-Arg-Asn)−NH−C(=O)−CH=CH_2 (abbreviated throughout as Acr-(VRN)-PEG-(VRN)-Acr).

A 23% solution of the macromer described above was prepared in HEPES-buffered saline (pH 7.4, 10 mM) with 900 ppm of 2,2-dimethoxy-2-phenylacetophenone as a long wavelength ultraviolet photoinitiator and 1500 ppm of *N*-vinylpyrrolidone, used as a polymerizable solvent for the photoinitiator, as described elsewhere.[31−33] The precursor was pipetted into molds and exposed to long wavelength ultraviolet light (10 mW/cm^2, 365 nm, 30 s exposure) to form hydrogel disks which were initially 5 mm in diameter and 2 mm thick. The initial wet weight of each disk was determined. The disks were then placed into 2 mL of HEPES-buffered saline for 24 h to allow them to reach their presumed equilibrium swelling levels, at which time their wet weight was determined. The disks were then placed in 2 mL of HEPES-buffered saline which also contained 0.2 mg/mL sodium azide (to prevent any bacterial growth, which would introduce additional protease sources) and 1 mM $CaCl_2$ (required for collagenase activity), either without enzyme, or with collagenase (Sigma; at 0.2 or 2 mg/mL), or with plasmin (Sigma; at 0.2 or 2 U/mL) to commence degradation. At predetermined time points, the wet weights of the disks were determined, and the buffer or enzyme solution was replaced.

Results. The synthesis of telechelic macromers Acr-(APGL)-PEG-(APGL)-Acr and Acr-(VRN)-PEG-(VRN)-Acr was accomplished with standard synthetic methodology for peptide synthesis, using DCC activation and Fmoc protection. Each cycle of addition of an amino acid residue and deprotection was found to proceed to greater than 90% completion, by UV spectroscopy for Fmoc and the ninhydrin assay for amine groups. The GPC analysis demonstrated elution at approximately 6000 g/mol for the parent PEG, while the APGL-containing and VRN-containing macromers eluted at a molecular weight of approximately 6400 g/mol. The degree of acrylation was found to be approximately 73% by proton NMR in the APGL-containing macromer and 76% in the VRN-containing macromer.

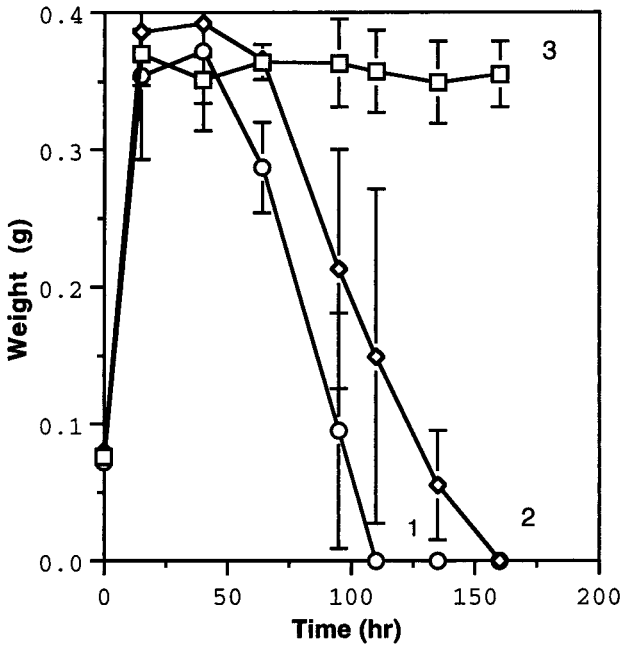

Figure 1. Enzymatic degradation of a hydrogel material formed by photopolymerization of Acr-(APGL)-PEG-(APGL)-Acr in the presence of 2 mg/mL collagenase (curve 1) or 0.2 mg/mL collagenase (curve 2), but not in the presence of 0.2 U/mL plasmin (curve 3). Each point represents the mean of four samples, and the standard error of the mean is shown.

Precursor solutions of both macromers readily formed cross-linked hydrogels under photoinitiation. These hydrogels formed in a manner similar to that as has been observed previously in telechelic PEG diacrylates containing oligoesters as hydrolytically degradable B blocks in the BAB block copolymer or containing no degradable B block at all.[31−33] In previous work, extents of incorporation of the PEG acrylate macromers into the gel phase were observed to be approximately 74% under the conditions employed in this study.[34]

The hydrogel formed from the Acr-(APGL)-PEG-(APGL)-Acr precursor swelled to a water content of approximately 86% at equilibrium (based on the mass of polymer in the sample and the wet weight after 24 h swelling), and the hydrogel formed from the Acr-(VRN)-PEG-(VRN)-Acr precursor swelled to approximately 89%, indicating that the characteristics of the networks were similar (Figures 1 and 2). Equilibrium water contents of approximately 94% were observed in similar hydrogels formed from PEG diacrylates (MW 8000) lacking degradable B blocks,[34] indicating that the incorporation of the rather bulky oligopeptide B blocks did not dramatically alter the polymerization of the macromer to form a cross-linked hydrogel network.

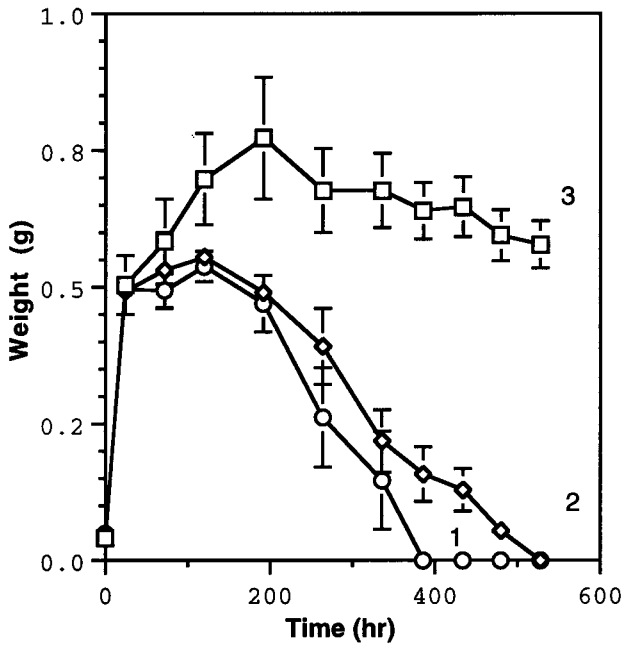

Figure 2. Enzymatic degradation of a hydrogel material formed by photopolymerization of Acr-(VRN)-PEG-(VRN)-Acr in the presence of 2 U/mL plasmin (curve 1) or 0.2 U/mL plasmin (curve 2), but not in the presence of 0.2 mg/mL collagenase (curve 3). Each point represents the mean of four samples, and the standard error of the mean is shown.

Both hydrogel materials were demonstrated to degrade readily in the presence of the targeted protease but remain stable in the presence of the other tested protease. The hydrogel material formed by photopolymerization of Acr-(APGL)-PEG-(APGL)-Acr was found to be specifically degraded by collagenase. In Figure 1, the degradation of the hydrogel disks in 2 mg/mL collagenase is shown in curve 1 and in 0.2 mg/mL collagenase in curve 2, while curve 3 demonstrates the lack of degradation in 0.2 U/mL plasmin. Degradation was not observed in the absence of proteases (data not shown). Similarly, the hydrogel formed by photopolymerization of Acr-(VRN)-PEG-(VRN)-Acr macromer was found to be specifically degraded by plasmin. In Figure 2, the degradation of these hydrogel disks in 2 U/mL plasmin is shown in curve 1 and in 0.2 U/mL in curve 2, while curve 3 demonstrates the substantial lack of degradation in 0.2 mg/mL collagenase.

Discussion. Telechelic block copolymers of PEG and short oligopeptides can be synthesized so that the resulting hydrogels are specifically degraded by targeted proteases involved in cell migration. This has been demonstrated with collagenase, targeting the peptide APGL, and with plasmin, targeting VRN. The ability to control protease selectivity lies in the design of the oligopeptide domain, such as its sequence and size, as discussed below. The overall structure of the macromer, i.e., with a central block of PEG, flanking blocks of the oligopeptides, and polymerizable acrylate groups at the chain termini, was appropriate for permitting both polymerization and proteolysis. The bulkiness of the oligopeptide block did not seem to inhibit polymerization of the macromer in the precursor solution, and the bulkiness of the PEG chain and the localization of the oligopeptide blocks adjacent to the cross-linking sites did not seem to inhibit proteolysis. Furthermore, on the time scale of degradation explored herein (more than 10 days) degradation was not observed in the absence of proteases. Over longer durations, one would expect some level of nonenzymatic degradation by hydrolysis of the ester link between the PEG and the oligopeptide blocks; one could limit this by starting from a PEG diamine, rather than a PEG diol, thus obtaining a more hydrolytically stable amide link between these two blocks.

Proteolytically degradable hydrogels may have especially useful properties in tissue engineering and wound healing.[35,36] When cells migrate and proliferate in vivo, either naturally in development or in wound healing, they do so by enzymatically creating three-dimensional pathways for their own migration;[3,26] it should be possible to take advantage of this biological phenomenon by designing materials to be sensitive to the enzymatic activities associated with cell migration. Such biological phenomena are not exploited by nonenzymatically degraded biomaterials such as polyesters of glycolic, lactic, or ϵ-caproic acid.[31] By contrast, these phenomena are exploited by biomaterials based on biologically derived precursors, such as proteins. For example, fibrin glues, formed from cryoprecipitated blood plasma, are resorbed by degradation via cellularly produced fibrinolytic enzymes.[37]

One advantage of employing biomimetic approaches in developing totally synthetic biomaterials that are enzymatically degradable lies in the control of material properties and the resulting biological response. In protein-based materials, such as fibrin glues, the rate of biomaterial resorption is controlled via the degree of cross-linking or the addition of protease inhibitors.[37] By contrast, the use of totally synthetic materials would permit the design of resorption rates based on the specific peptide selected, e.g., by the independent design of K_m and k_{cat} to respond to cellular signals in different biological contexts. This can presumably be accomplished by design of the identity of the oligopeptide domain, its length, and the length of spacer domains separating it from the neighboring PEG chain, opening a path to careful molecular engineering of the substrate regions within the hydrogel. For example, in the context of collagenase-sensitive materials, the kinetics of collagenase cleavage of synthetic peptides has been examined by Netzel-Arnett et al.[22] in a study of the effects of substrate length. Relative to the collagenase-sensitive site in α1(I) collagen spanning from P_4 to P'_4 (100%), truncation of the sequence to span from P_4 through P'_3 reduced activity (measured by k_{cat}/K_m) to 68%, truncation to span from P_4 to P'_2 reduced it to 13%, and truncation further to span from P_4 to P'_1 reduced the relative rate of cleavage to less than 5%. One should be able to exploit this and related information in the design of gels that respond faster or slower to biological signals involved in wound healing.

There may be many applications of synthetic materials that respond to biological signals by proteolytic degradation. For example, the group of Kopecek has developed a family of polymer-conjugated drugs, in which a low molecular weight drug is attached to a high molecular weight polymeric carrier via an oligopeptide that is cleaved by intracellular proteases; this results in release of the drug only after the conjugate has entered the cell, providing precise intracellular targeting.[38,39] The ultimate goal of the work reported in this paper is the development of cell-responsive materials for wound healing and tissue engineering applications. Such applications include the promotion of peripheral

nerve regeneration, the control of wound healing of arteries after injury due to procedures such as balloon angioplasty, and cell transplantation into scaffolds that can be remodeled by the incorporated cells during tissue synthesis. In the context of arterial healing, for example, previous studies have demonstrated in animal models that PEG diacrylate gels with oligo(lactic acid) nonenzymatically hydrolyzed B blocks can be interfacially photopolymerized in situ on the surface of arteries injured by balloon angioplasty and can dramatically improve the wound healing outcome by acting as a barrier against thrombosis.[40,41] The use of enymatically degradable materials in such an application might permit the endothelial cells that are involved in resurfacing the injured artery during healing to induce the local and sequential removal of this therapeutic barrier, thus providing the artery with the desired endothelial surface.

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