



Maleimide–thiol coupling of a bioactive peptide to an elastin-like protein polymer

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ABSTRACT

Recombinant elastin-like protein (ELP) polymers display several favorable characteristics for tissue repair and replacement as well as drug delivery applications. However, these materials are derived from peptide sequences that do not lend themselves to cell adhesion, migration, or proliferation. This report describes the chemoselective ligation of peptide linkers bearing the bioactive RGD sequence to the surface of ELP hydrogels. Initially, cystamine is conjugated to ELP, followed by the temperature-driven formation of elastomeric ELP hydrogels. Cystamine reduction produces reactive thiols that are coupled to the RGD peptide linker via a terminal maleimide group. Investigations into the behavior of endothelial cells and mesenchymal stem cells on the RGD-modified ELP hydrogel surface reveal significantly enhanced attachment, spreading, migration and proliferation. Attached endothelial cells display a quiescent phenotype.

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1. Introduction

Experimental small-diameter vascular grafts underperform due to thrombogenicity of the luminal surface and neointimal hyperplasia attributed to a mechanical mismatch between synthetic materials and arterial tissue [1,2]. Recombinant elastin-like protein (ELP) materials have emerged in principle as attractive biomaterials for vascular applications because they are based on amino acid sequences from elastin, a key structural protein of the native vasculature [3]. We have recently described triblock ELP polymers with hydrophilic, elastomeric midblock sequences flanked by self-associating, hydrophobic endblocks in an ABA triblock format [4–6]. Notably, triblock ELPs are highly soluble under cool, aqueous conditions, but form elastomeric gels when solutions are raised above an inverse transition temperature. Under these conditions the more hydrophobic endblocks coacervate to form physical crosslinks while the midblocks remain elastomeric and solvated. As an example, *LysB10*, a 209 kDa triblock ELP, has an inverse transition temperature of 13 °C [6]. Due to the inclusion of lysine residues at the block interfaces, *LysB10* can be crosslinked by chemical strategies that reinforce physical crosslinking. Our studies have shown that triblock ELPs are non-thrombogenic [7], can be highly

biostable [8], permit controlled drug release [9] and can be used in protein-based composites that mimic native artery mechanics [10]. Despite these advantages, the peptide sequences typically employed in ELP design do not support cell adhesion.

Poor patency rates of synthetic polymers have motivated strategies to promote luminal endothelialization [11–17], often through covalent tethering of biomolecules [18]. Because these reactions are typically between the amino acid side chains and activated surface functional groups, uncontrolled or non-specific covalent binding often results. Herein, we describe maleimide–thiol chemistry for hydrogel surface biofunctionalization. The high degree of specificity and reactivity of sulfhydryl groups with maleimide moieties to form stable thioether bonds has been exploited extensively for the construction of immobilized antibodies, enzymes and peptide-conjugated haptens [19–22]. Maleimide reacts approximately 1000 times faster with thiols than with amines at neutral pH and below, and this reaction is widely used for conjugation of cysteine-containing peptide and proteins [23–25].

The RGD sequence was explored as a model peptide conjugate, given its ubiquitous nature in extracellular matrix (ECM) proteins, including fibronectin, vitronectin, fibrinogen, von Willebrand factor, as well as collagen, and its ability to bind numerous integrins, notably $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [26]. Several biomaterials have been functionalized with RGD, including polymers such as poly(ethylene glycol) (PEG) hydrogels [27,28], polyacrylamide [29,30], poly(2-hydroxyethyl methacrylate) [31,32], poly(lactic acid-co-lysine) [34,35], poly(propylene fumarate) [33,34] and

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polyurethanes [35,36]; and biopolymers, including collagen [37,38], fibrin [14,39], hyaluronic acid [40,41], alginate [42,43], dextran [44,45], ELPs [46–48] and silk-like proteins [49].

2. Materials and methods

2.1. Reagents, antibodies and cells

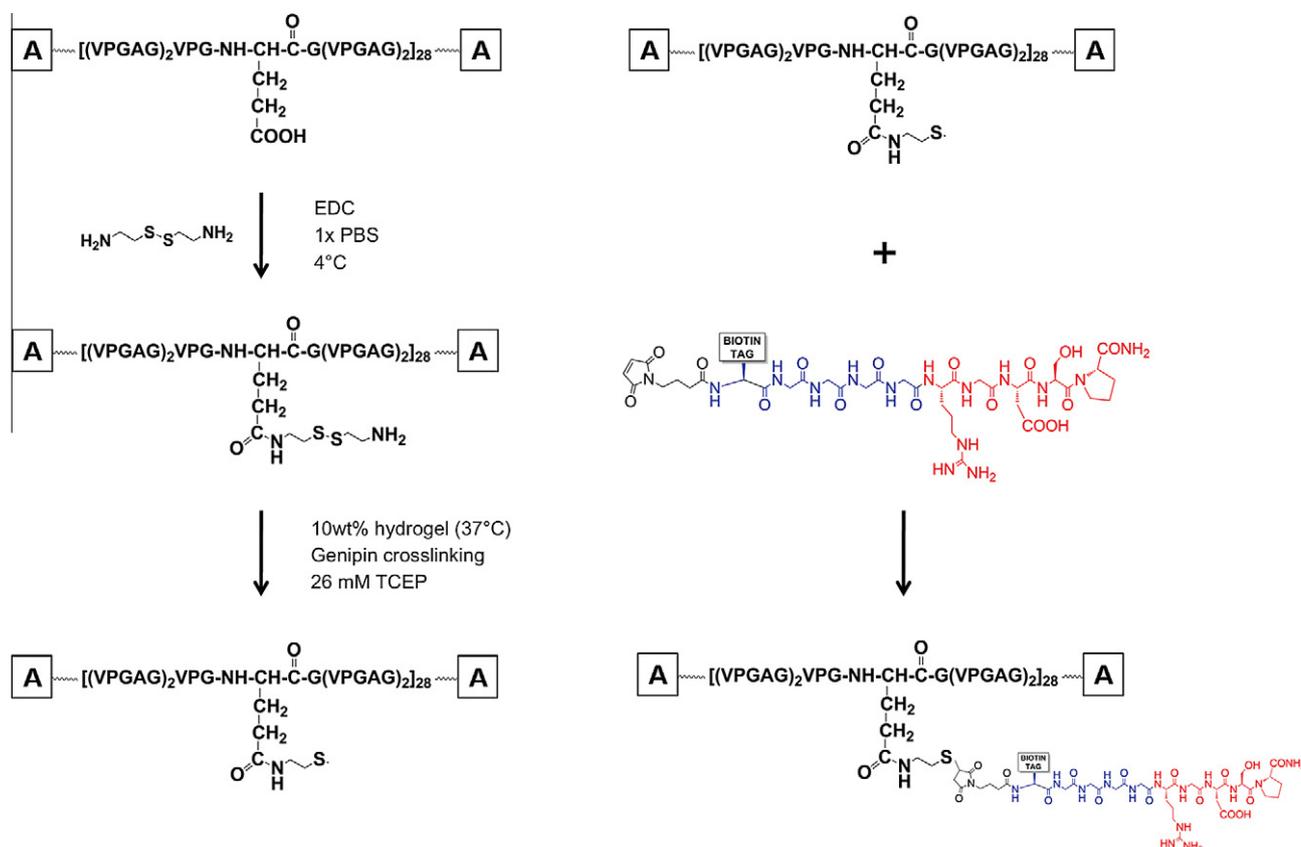
All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. The peptide sequences GRGDSP and GRGESP were synthesized by AnaSpec (Fremont, CA). Porcine mesenchymal stem cells (pMSCs) were a kind gift from Dr. Steven Stice (University of Georgia). The biosynthetic strategy for the expression and purification of the recombinant ELP triblock polymer, *LysB10*, has been described previously [6].

2.2. Cystamine modification of *LysB10*

LysB10 was chemically modified utilizing aqueous carbodiimide chemistry (Scheme 1) [24]. Cystamine (Sigma Aldrich) was added to the solution at 20-fold molar excess to a cooled solution of *LysB10* (10 mg ml⁻¹, 4 °C PBS), followed by *N*-(3-dimethylamino-propyl)-*N'*-ethylcarbodiimide (EDC) at 5-fold molar excess relative to cystamine. After stirring (72 h, 4 °C) cystamine-modified *LysB10* polymer was purified by dialysis and lyophilization (81% yield).

2.3. Solid-phase peptide synthesis

The RGDSP (Arg–Gly–Asp–Ser–Pro) peptide was synthesized manually on a Rink amide resin using the standard Fmoc amino acid coupling strategy [50]. Briefly, Fmoc-Pro-Rink amide resin (1.0 g, 0.45 mmol g⁻¹) was loaded into a fritted column equipped with a plastic cap. The resin was swelled by stirring gently with 20 ml dichloromethane (DCM) for 10 min and filtered. This procedure was repeated with 4 × 20 ml portions of DCM and followed by 4 × 20 ml portions of dimethylformamide (DMF). Deprotection of Fmoc was performed with 20% piperidine in DMF (2 × 10 ml) for 20 min. The protected amino acids used in the coupling sequences are Fmoc–Ser–OH, Fmoc–Asp(tBu)–OH, Fmoc–Arg(Pbf), Fmoc–Glu(biotinyl–PEG)–OH and 3-maleimido propionic acid. Except for the Fmoc–Glu(biotinyl–PEG)–OH coupling reaction, every successive coupling was performed with four equivalents of Fmoc-amino acid preactivated for 5 min with HBTU/HOBt in 10 ml DMF. The Fmoc–Glu(biotinyl–PEG)–OH reaction was performed with PyBOP/HOBt as the coupling reagent. The progress of the deprotection/coupling was followed at every cycle by performing the ninhydrin test using the Kaiser kit. Cleavage/deprotection of the peptide from the resin was accomplished by gently stirring in 20 ml of a 1/2/2/95 of water/ethanedithiol/triethylsilane/trifluoroacetic acid for 2 h and filtering. The peptide was obtained as a yellow syrup, which was precipitated as a white solid by adding the cold ether (50 ml). The precipitated peptide was collected



Scheme 1. Reaction scheme of *LysB10* modification and peptide coupling. Amide bond formation was mediated by the carbodiimide through the carboxylic group of the amino acid and the amine of cystamine, resulting in thiolated *LysB10*. The plastic domains of *LysB10* are represented as “A” endblocks. Hydrogel formation was achieved by placing 10 wt.% thiol-*LysB10* solution at 37 °C, well above the transition temperature of the protein polymer. Lysine residues of the protein polymer were crosslinked with a 6 mg ml⁻¹ genipin solution for 24 h, followed by stringent PBS rinsing to remove all genipin. The thiol groups were reduced with the addition of 26 mM Tris(2-carboxyethyl)phosphine (TCEP) to form free sulfhydryls. After rinsing the gels with three 20 min PBS washes, thiol-reactive peptide linker was incubated for 2 h at room temperature to form a thioether bond with the protein polymer. Peptides were generated via solid-phase synthesis, with key features incorporated in the design. The N-terminus of the molecule contains the thiol-reactive maleimide linker (black). Four glycine residues (blue) act as a spacer between the cell-binding RGD domain (red) and the remaining sequence to facilitate ligand–integrin presentation. A biotinyl–PEG₃ tag was incorporated into the peptide for detection of the molecule.

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