Journal of Controlled Release 191 (2014) 71–81

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

# Avidity-controlled hydrogels for injectable co-delivery of induced pluripotent stem cell-derived endothelial cells and growth factors

Widya Mulyasasmita [a], Lei Cai [b,c], Ruby E. Dewi [b], Arshi Jha [c,d], Sabrina D. Ullmann [e], Richard H. Luong [f], Ngan F. Huang [c,d,g], Sarah C. Heilshorn [a,b,c,*]

[a] Department of Bioengineering, Stanford University, Stanford, CA, USA
[b] Department of Materials Science and Engineering, Stanford University, Stanford, CA, USA
[c] Stanford Cardiovascular Institute, Stanford University, Stanford, CA, USA
[d] Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA
[e] Department of Chemistry, RWTH Aachen University, Aachen, Germany
[f] Department of Comparative Medicine, Stanford University, Stanford, CA, USA
[g] Division of Cardiovascular Medicine, Stanford University, Stanford, CA, USA

ARTICLE INFO

*Article history:*
Received 11 April 2014
Accepted 8 May 2014
Available online 18 May 2014

*Keywords:*
iPSC
Hydrogel
Protein engineering
VEGF
Endothelial cell

ABSTRACT

To translate recent advances in induced pluripotent stem cell biology to clinical regenerative medicine therapies, new strategies to control the co-delivery of cells and growth factors are needed. Building on our previous work designing Mixing-Induced Two-Component Hydrogels (MITCHs) from engineered proteins, here we develop protein–polyethylene glycol (PEG) hybrid hydrogels, MITCH-PEG, which form physical gels upon mixing for cell and growth factor co-delivery. MITCH-PEG is a mixture of C7, which is a linear, engineered protein containing seven repeats of the CC43 WW peptide domain (C), and 8-arm star-shaped PEG conjugated with either one or two repeats of a proline-rich peptide to each arm (P1 or P2, respectively). Both 20 kDa and 40 kDa star-shaped PEG variants were investigated, and all four PEG-peptide variants were able to undergo a sol–gel phase transition when mixed with the linear C7 protein at constant physiological conditions due to noncovalent heterodimerization between the C and P domains. Due to the dynamic nature of the C–P physical crosslinks, all four gels were observed to be reversibly shear-thinning and self-healing. The P2 variants exhibited higher storage moduli than the P1 variants, demonstrating the ability to tune the hydrogel bulk properties through a biomimetic peptide-avidity strategy. The 20 kDa PEG variants exhibited slower release of encapsulated vascular endothelial growth factor (VEGF), due to a decrease in hydrogel mesh size relative to the 40 kDa variants. Human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) adopted a well-spread morphology within three-dimensional MITCH-PEG cultures, and MITCH-PEG provided significant protection from cell damage during ejection through a fine-gauge syringe needle. In a mouse hindlimb ischemia model of peripheral arterial disease, MITCH-PEG co-delivery of hiPSC-ECs and VEGF was found to reduce inflammation and promote muscle tissue regeneration compared to a saline control.



## 1. Introduction

We introduce a new family of injectable hydrogels formed through the dynamic hetero-assembly of a linear, engineered protein with a star-shaped, peptide-modified PEG molecule that takes advantage of biomimetic avidity to fine-tune the hydrogel properties. Following the first report of Mixing-Induced Two-Component Hydrogels (MITCHs), which were composed entirely of linear, engineered proteins [1], the strategy was expanded by several groups to demonstrate the successful mixing-induced gelation of star-shaped, peptide-modified PEG molecules using a variety of peptide binding domains [2–6]. In all of these systems, gelation is induced by noncovalent peptide hetero-assembly when the individual hydrogel components are mixed together. Because the mechanical properties of MITCH systems are directly related to the concentration and binding affinity of the hetero-assembled crosslinks [1,7], we hypothesized that presenting dimers of binding ligands in close proximity would result in a dramatic lowering of the apparent equilibrium dissociation constant, $K_{d,app}$, and hence increase the number of hetero-assembled crosslinks. This gelation mechanism mimics the evolved strategy of avidity, *i.e.* the combined enhancement of multiple weak affinity interactions. At a molecular level, avidity can be mechanistically explained by considering the dynamic, noncovalent binding of a single protein receptor to a single ligand within a larger array of ligands. Although the protein receptor will bind and unbind

* Corresponding author at: 476 Lomita Mall, McCullough Room 246, Stanford University, Stanford, CA 94305-4045, USA. Tel.: +1 650 723 3763; fax: +1 650 498 5596.
*E-mail address:* heilshorn@stanford.edu (S.C. Heilshorn).

http://dx.doi.org/10.1016/j.jconrel.2014.05.015
0168-3659/

the ligand with the kinetic rate constants, $k_{on}$ and $k_{off}$, the likelihood of the unbound receptor quickly finding another ligand to rebind will increase as the ligand density increases. Therefore, the apparent $k_{on}$ also increases with ligand density, effectively lowering the apparent equilibrium dissociation constant. As a result, multiple weak binding interactions can appear to have a much higher effective binding energy. While this avidity-based, multivalent strategy has been widely applied in the field of nanobiotechnology for drug delivery, bioimaging, and biosensing applications [8–10], it has yet to be explored in peptide hetero-assembled hydrogels.

We further hypothesized that these avidity-controlled MITCH systems would be tunable carriers for the co-delivery of induced pluripotent stem cell-derived endothelial cells and growth factors. Human-induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) are ideal candidates for vascular therapy. Reprogrammed from somatic cells, iPSCs offer an abundant source of autologous cells that mitigate immunogenicity and ethical concerns [11]. In a murine hindlimb ischemia model for peripheral arterial disease (PAD), hiPSC-ECs injected into the ischemic calf muscle enhanced microvessel density and improved blood reperfusion by secreting angiogenic cytokines and incorporating into expanding endogenous microvasculature [12]. The clinical adoption of hiPSC-EC therapy is currently hampered by the rapid decline of transplanted cell viability, which necessitates multiple cell administrations for sustaining therapeutic efficacy [12]. Acutely, the number of viable cells plummets during injection due to membrane-disruptive extensional forces experienced in the syringe needle [13]. Pre-encapsulation of cells within a shear-thinning, self-healing physical hydrogel can provide significant protection from these mechanical forces [13–15]; therefore, we hypothesized that avidity-controlled MITCH systems may provide a similar protective effect.

Without the aid of a biomaterial carrier, efforts to enhance transplanted cell survival typically involve donor cell supplementation with biochemical factors that block apoptotic pathways associated with hypoxia and inflammatory insult [16,17]. Vascular endothelial growth factor (VEGF) is a promising drug for co-delivery with hiPSC-ECs into ischemic target tissues, since VEGF is known to promote endothelial cell proliferation, survival and migration. In addition to enhancing donor cell survival, VEGF is a potent pro-angiogenic signal that could augment hiPSC-EC paracrine signaling and improve blood reperfusion by coaxing endogenous revascularization. Similar to many soluble pro-survival factors [18], VEGF has a short *in vivo* circulating half-life (3 min in mice) [19]. Moreover, bolus delivery of VEGF results in a rapid dosage burst that produces leaky and aberrant vessels and off-target effects [20] instead of mature and stable vasculature. In a murine hindlimb ischemia model, optimal revascularization was achieved by a profile of high initial VEGF dosage, followed by steadily decreasing concentration over time [20]. Temporal regulation therefore underpins the safety and efficacy of cell and VEGF combinatorial therapy.

Previously, several types of hydrogel scaffolds have been used to deliver cells and angiogenic growth factors separately or in combination to increase vascularization in ischemic models [21–23]. These hydrogels include collagen–fibronectin [24], alginate [24,25], fibrin [26], gelatin [27], poly(lactide-co-glycolide) (PLGA) [28,29], and peptide amphiphiles [30]. A collagen–fibronectin gel that encapsulated VEGF-loaded alginate microparticles and endothelial cells was implanted into the gastrocnemius muscle of mice with induced hindlimb ischemia [24]. VEGF released from the microparticles increased the survival of the transplanted endothelial cells and enhanced muscle myoglobin expression, a sign of recovery from ischemia, compared to solely cell transplantation or VEGF delivery [24]. This synergistic angiogenic effect was also demonstrated by implanting fibrin scaffolds containing angiogenic growth factors and directly injecting bone marrow cells to the murine ischemic muscles [26]. While these implanted hydrogel scaffolds showed enhanced neovascularization by co-delivery of cells and growth factors, the gels were not injectable and required surgical implantation. Furthermore, these scaffolds, which are based on harvested biopolymers (*e.g.* collagen, fibronectin, fibrin), offer limited control over the material properties compared to engineered matrices.

To demonstrate the suitability of our newly developed avidity-controlled MITCH system for injectable co-delivery of hiPSC-ECs and VEGF, we synthesized and characterized a family of four hydrogels with tunable viscoelastic and diffusive properties. As proof of concept, we further evaluated the lead formulation in a preclinical murine hindlimb ischemia model of peripheral arterial disease. To the best of our knowledge, this work represents the first demonstration of avidity-controlled, injectable hydrogels for applications in regenerative cell and drug combination therapy.

## 2. Materials and methods

### 2.1. Synthesis and purification of C7 protein

The C7 recombinant protein polymer (see Fig. S1 for full amino acid sequence) was cloned, synthesized, and purified as reported previously [1]. In brief, the DNA sequence encoding the C7 block copolymer was cloned into the pET-15b vector (Novagen) and transformed into the BL21(DE3)pLysS *Escherichia coli* host strain (Life Technologies). Protein was expressed following isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, purified by affinity chromatography *via* the specific binding of N-terminal polyhistidine tag to Ni-nitrilotriacetic acid resin (Qiagen), buffer exchanged into phosphate-buffered saline (PBS), and concentrated by diafiltration across Amicon Ultracel filter units (Millipore) with 30 kDa Molecular Weight Cut-Off (MWCO). Protein identity and purity were confirmed by gel electrophoresis, MALDI-TOF mass spectrometry, and amino acid compositional analysis (data not shown).

C7 protein used in *in vivo* experiments was further subjected to lipopolysaccharide (LPS) removal by four cycles of phase separation and temperature transition extraction with Triton X-114. Residual Triton X-114 was removed by overnight incubation with Bio-Beads SM-2 Adsorbents (Biorad), and the PyroGene Recombinant Factor C Endotoxin Detection Assay kit (Lonza) was used to confirm the reduction of LPS levels to below 5 EU/mg in the final C7 protein solutions.

### 2.2. Conjugation of P1 and P2 peptides to 8-arm PEG

8-Arm polyethylene glycol vinyl sulfone (8-arm-PEG-VS) with nominal molecular weights of 20 and 40 kDa were purchased from Nanocs (Boston, MA). Peptides P1 (EYPPYPPPPYPSGC, 1563 Da) and P2 (EYPPYPPPPYPSGGGGGEYPPYPPPPYPSGC, 3234 Da) were purchased through custom peptide synthesis from Genscript Corp (Piscataway, NJ, USA) and confirmed to have purity of 92–98% by HPLC. All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless otherwise noted. A Michael-type addition of P1 or P2 to 8-arm-PEG-VSs was conducted in a Schlenk tube in the presence of tris(2-carboxyethyl) phosphine (TCEP). Peptides (P1 or P2), 8-arm-PEG-VS (20 kDa or 40 kDa), and TCEP were dissolved at a molar ratio of thio:VS:TCEP = 1.5:1:0.05 in 0.3 M triethanolamine (TEA) solution and pH was adjusted to 8.0. The reaction solution was then degassed, flushed with nitrogen, and maintained in a 37 °C incubator for 24 h. The solution was then lyophilized and washed with chloroform to remove unreacted PEG. The precipitate was redissolved and dialyzed into deionized water, using 10 kDa MWCO dialysis tubing. A second lyophilization step yielded the final 8-arm-PEG–peptide conjugate products as white powder, with overall conjugation efficiency of ~80%.

The chemical structures of the purified 8-arm-PEG–peptide conjugates were confirmed by $^1$H Nuclear Magnetic Resonance (NMR) spectrometry, acquired on a Varian Inova 500 MHz NMR spectrometer. Deuterium oxide containing a trace amount of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (Cambridge Isotope Laboratories, Andover, MA) was used as a solvent. Peptide conjugation efficiency was quantified from the ratio of the area under the tyrosine doublets to the peak area corresponding to the PEG backbone. Compositional