Biomaterials 113 (2017) 170–175

Contents lists available at ScienceDirect

Biomaterials

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

Protease-degradable microgels for protein delivery for vascularization

Greg A. Foster [a, b], Devon M. Headen [a, b], Cristina González-García [a, b, c], Manuel Salmerón-Sánchez [a, b, c], Haval Shirwan [d], Andrés J. García [a, b, *]

[a] Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA
[b] Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA
[c] School of Engineering, Division of Biomedical Engineering, University of Glasgow, Glasgow, Scotland, UK
[d] Department of Microbiology and Immunology, University of Louisville, Louisville, KY, USA

ARTICLE INFO

Article history:
Received 4 August 2016
Received in revised form
19 October 2016
Accepted 27 October 2016
Available online 28 October 2016

Keywords:
VEGF
Microfluidics
Biomaterials
Hydrogels
Protein delivery

ABSTRACT

Degradable hydrogels to deliver bioactive proteins represent an emerging platform for promoting tissue repair and vascularization in various applications. However, implanting these biomaterials requires invasive surgery, which is associated with complications such as inflammation, scarring, and infection. To address these shortcomings, we applied microfluidics-based polymerization to engineer injectable poly(ethylene glycol) microgels of defined size and crosslinked with a protease degradable peptide to allow for triggered release of proteins. The release rate of proteins covalently tethered within the microgel network was tuned by modifying the ratio of degradable to non-degradable crosslinkers, and the released proteins retained full bioactivity. Microgels injected into the dorsum of mice were maintained in the subcutaneous space and degraded within 2 weeks in response to local proteases. Furthermore, controlled release of VEGF from degradable microgels promoted increased vascularization compared to empty microgels or bolus injection of VEGF. Collectively, this study motivates the use of microgels as a viable method for controlled protein delivery in regenerative medicine applications.

1. Introduction

Synthetic hydrogel microparticles (microgels) have broad biomedical applications including cell encapsulation and transplantation [1–8], wound healing [9], imaging tools [10], and protein and drug delivery [11–15]. Microgels offer additional advantages to the attributes of bulk hydrogels for cell and protein delivery, including delivery via catheters or injection via small diameter needles, which minimizes complications associated with surgery (e.g. trauma, infection, scarring), and preserves native tissue structure without *in situ* gelling considerations that often limit biomedical applications of bulk hydrogels. Furthermore, when appropriately sized, microgels conform to the geometry of the application site, which facilitates uniform distribution of biomolecules to target sites. Importantly, microgels with different characteristics (e.g., different proteins, release rates) can be synthesized in separate batches and simple co-delivery of the microgels in the desired ratios will result in a "mosaic" formulation resulting in complex or multi-component materials.

Of various synthesis routes available to generate synthetic microgels, microfluidics-based polymerization is particularly well-suited for preparing microgels containing proteins and cells because of the aqueous, cytocompatible nature and precise control over particle size of this continuous process [7]. Microgels for protein delivery rely on passive diffusion of the protein through a non-degradable microgel network, and therefore the release kinetics are solely dictated by protein size and microgel mesh size [16]. This inability to modulate protein delivery rate severely hinders the application of microgels to regenerative medicine, immunoengineering, and cancer therapy. We present a strategy to engineer synthetic microgels with protease-degradable crosslinks and tunable protein release kinetics. Furthermore, we demonstrate that these protease-degradable microgels promote *in vivo* vascularization by controlled release of vascular endothelial growth factor (VEGF) and complete degradation of microgels that allows for tissue ingrowth and remodeling.

* Corresponding author. Woodruff School of Mechanical Engineering, 315 Ferst Dr NW, Atlanta, GA 30332, USA

E-mail address: andres.garcia@me.gatech.edu (A.J. García).

http://dx.doi.org/10.1016/j.biomaterials.2016.10.044
0142-9612/

2. Materials and methods

2.1. Microfluidic device fabrication

PDMS microfluidic flow focusing devices were fabricated using soft lithography from silicon and SU8 masters. Devices were plasma treated and then bonded directly to glass slides. Microfluidic devices were then heated to 110 °C for 30 min to improve PDMS-glass sealing. Prior to use, devices were infused with Aquapel™ for 30 s and then purged with nitrogen to render surfaces hydrophobic.

2.2. 4-Arm poly(ethylene glycol)-maleimide (PEG-4MAL) microgel generation

PEG-4MAL (20 kDa, Laysan Bio) was dissolved in phosphate-buffered saline (PBS) at 5% (w/v) then filtered through a 40 μm cell strainer (Corning). For experiments involving the injection of microgels *in vivo*, microgels were functionalized with GRGDSPC (RGD, Genescript). PEG-4MAL was reacted with 2.0 mM RGD for 30 min at 37 °C to create RGD-functionalized macromer. For all other experiments, RGD was not used in the formation of microgels. Crosslinker solutions (DTT (Sigma) or GCRDVPMSMRGGDRCG (VPM, Genescript) or combinations of both) were prepared at predetermined molar concentrations and then adjusted to a pH of 4.5 to slow down gelation kinetics in order to prevent the device from clogging. PEG-4MAL and crosslinker were then infused into the flow-focusing microfluidic device to form polymer droplets. Droplets were formed within an oil solution consisting of light mineral oil (Sigma) mixed with 2% SPAN80 (Sigma) and then collected into a 15 mL conical tube (Falcon). After formation, microgels were washed in PBS five times by centrifugation to remove mineral oil and surfactant.

2.3. Microgel degradation

Two hundred microgels were loaded into each well of a 96-well plate. Collagenase or PBS was then added to each well and microgels were incubated at 37 °C for 20 h. After incubation with protease or PBS, images of each well were acquired using a fluorescent microscope and the total number of microgels in the well was quantified.

2.4. Protein release kinetics

Prior to microgel formation, PEG-4MAL was reacted with AlexaFluor488-labeled IgG (rat anti-mouse, Thermo Fisher), AlexaFluor555-labeled IgG (rat anti-mouse, Thermo Fisher), or VEGF165 homodimer (Thermo Fisher) pre-labeled with NHS-Dylight488 (Thermo Fisher). All proteins were reacted with PEG-4MAL at 20 μg/mL for 30 min at 37 °C protected from light. After washing, 100 μL of 200 μm diameter microgels were added to transwells with 8 μm pore sizes in a 48 well plate (Corning) then treated with 3.9 or 39 units/mL of type 1 collagenase in 500 μL of PBS (Worthington). The microgels were then maintained in an incubator at 37 °C with gentle shaking. At indicated time points, the supernatant was sampled and analyzed on a plate reader (Biotek). Images of the microgels were acquired on an inverted microscope (Nikon TE 300) with a fluorescent camera (Hamamatsu Orca ER II).

2.5. VEGF bioactivity assay

We have previously shown that PEGylation of VEGF homodimer primarily results in a VEGF molecule conjugated to two PEG-4MAL macromers [17]. To confirm PEGylated VEGF maintained bioactivity, human umbilical vein endothelial cells (HUVEC, Lonza) were grown in endothelial growth media (EGM-2, Lonza) and synchronized in growth factor free basal media (EBM-2, Lonza) with 1% fetal bovine serum overnight followed by addition of VEGF, PEG-4MAL conjugated VEGF, PEG-MAL only, or EGM-2 for 24 h. Cell metabolism was assayed by the CellTiter 96 MTS Aqueous Cell Proliferation Assay (Promega). To confirm VEGF released from microgels maintained bioactivity, microgels containing VEGF were incubated in MMP-2 (50 nM) (R&D Systems) for 30 min at 37 °C followed by addition of TIMP-1 (50 nM). HUVEC were then exposed to released VEGF (100 ng/mL) or soluble VEGF (100 ng/mL) for 24 h and cell metabolism was assayed.

2.6. Microgel vascularization

To track microgel retention *in vivo*, RGD was conjugated with Dylight750 for IVIS imaging or Dylight555 for microscopy imaging then tethered to PEG-4MAL macromer. Under protocols approved by Georgia Tech's Institutional Animal Care and Use Committee, C57BL/6J mice (Jackson Labs) were anesthetized with 2.5% isoflurane during microgel injection and image acquisition. Backs of mice were shaved, dilapidated with Nair™, and sterilized with 70% ethanol. A 1 mL syringe with a 23 gauge 0.5″ needle was loaded with 100 μL of microgels. The tip of needle was then inserted into the subcutaneous space of the dorsum and microgels were slowly injected, taking care not to disturb the native tissue structures. A total of 16 mice received two of the following microgel formulation chosen for this study: VPM + VEGF, VPM − VEGF, DTT + VEGF, VPM + sVEGF. Experimental groups were designed such that 4 samples were used for each group. IVIS Spectrum CT (Perkin Elmer) imaging system was used to track microgel position and persistence over time. At 14 day, following injection, functional vasculature was labeled by perfusing anesthetized mice with 1.0 mg/mL Dylight649-labeled tomato lectin (Vector Labs) via tail vein injection. To wash out excess fluorescent lectin, mice were perfused with saline solution. Mice were then euthanized with CO_2 and the regions of the skin where microgels were injected were excised. Microgels and vasculature were imaged using a confocal microscope (Nikon Ti-E with Perfect Focus System and C2-Plus Confocal System) and analyzed with ImageJ software.

3. Results

3.1. Generation of microgels using microfluidics

To engineer synthetic microgels, we used a PEG-4MAL macromer, which is crosslinked into a network via a Michael-type addition reaction with thiols. The PEG-4MAL platform outperforms other PEG-based polymers in generating structurally defined hydrogels with stoichiometric incorporation of ligands and improved crosslinking efficiency [17]. In addition, PEG-4MAL exhibits minimal local and systemic inflammation and toxicity and is rapidly excreted in the urine [18], important criteria for *in vivo* applications. We designed a microfluidic flow focusing device to produce droplets of PEG-4MAL and crosslinker (Fig. 1a). Three independent flow inlets (PEG-4MAL, crosslinker, and mineral oil containing SPAN80 surfactant) were used to produce droplets. After the PEG-4MAL and crosslinker flow streams merge, the solution is focused into an oil-covered droplet where it crosslinks and is then collected at the outlet (Video S1, Supporting Information). Microgels of defined diameters with homogeneous size distribution can be simply produced by changing the inlet flow rates and nozzle size (Fig. 1b,c). To generate protease-degradable microgels, we used the crosslinking peptide GCRDVPMSMRGGDRCG (VPM), which is rapidly cleaved by matrix metalloproteinase (MMP)-1 and MMP-2 proteases [19]. To confirm protease-dependent degradation, we