Biomaterials 101 (2016) 199–206

Contents lists available at ScienceDirect

Biomaterials

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

Controlling the kinetics of thiol-maleimide Michael-type addition gelation kinetics for the generation of homogenous poly(ethylene glycol) hydrogels

Nicole J. Darling, Yiu-Sun Hung, Shruti Sharma, Tatiana Segura*

Chemical and Biomolecular Engineering Department, University of California Los Angeles, 420 Westwood Plaza, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form
14 May 2016
Accepted 30 May 2016
Available online 2 June 2016

Keywords:
Maleimide
Hydrogel
Rheology
Peptide design
Gelation kinetics

ABSTRACT

The development of synthetic hydrogels analogs for the extracellular matrix has proven a useful and important tool to study the role of specific signals on biological outcomes *in vitro* and to serve as scaffolds for tissue repair. Although the importance of physical properties (e.g. microstructure and stiffness) in the micro and nano scale on cell fate has been widely reported, bulk modulus measurements are typically used to characterize hydrogels. Thus, the physical properties of hydrogels have not been widely tested for their controlled physical properties in the nano and micron scales. In this report, we show that although fast Michael-type addition crosslinked hydrogels appear uniform by bulk modulus readings and visual inspection, they are non-uniform in the micron scale, with high and low crosslinking regions. Further, we show that these regions of high and low crosslinking result in differences in cellular behavior. Since these regions are random in density and shape, this leads to misleading cellular responses. These inconsistences are most widely observed when the gel forms faster than the material can be mixed. This study slows the gelation rate of thiol-maleimide cross-linked hydrogels in order to overcome the cellular response variability between batches.

1. Introduction

The development of poly(ethylene glycol) (PEG)-based synthetic hydrogels as an analog extracellular matrix for cell culture *in vitro* and tissue regeneration *in vivo* has been widely used and accepted [1–4]. During the last decade the importance of physical properties such as, mechanical stiffness and topography of the cellular environment have been recognized to impact the cellular behavior and differentiation [5–7]. Thus, to effectively study the cellular response to the material each input parameter must be controllable and well defined. In the present study, we report the importance of balancing the gelation rate with the mixing rate for obtaining repeatable hydrogel microstructures within the widely used Michael-type addition hydrogels. We find that fast gelation kinetics lead to inefficient mixing, heterogeneous gelation, and inconsistent cell responses to the hydrogel. In particular, the fast gelation kinetics of the thiol-maleimide reaction lead to areas of low or high crosslinking that cannot be controlled unless the pH is changed to incompatible ranges. We further report on different approaches to slow down the thiol-maleimide reaction kinetics through reducing the reactivity of the thiol to result in controllable gel microstructure and cellular behavior.

Topographical and mechanical cues are strong cellular activators that can override biochemical cues [8,9]. Thus, synthetic microenvironments aiming to generate extracellular matrix mimics must be able to control the topographical and mechanical cues throughout the material. *In situ* forming Michael-type addition hydrogels are typically regarded as a homogenous polymer network environment; however, in these types of hydrogels functional groups begin crosslinking immediately upon mixing (nothing is preventing the addition reaction from taking place), generating micro domains within the gels. These domains become more prominent as the reaction speed becomes greater than the mixing speed. This is a key point that has largely been ignored as the gels are assumed to be homogeneous because their bulk properties are consistent from experiment to experiment and the gel macrostructure (upon visual inspection) is uniform. Herein, we focused on demonstrating the inconsistency of fast Michael-type addition gelation and improving microstructure homogeneity of thiol-maleimide Michael-type addition reaction hydrogels by

* Corresponding author.
E-mail address: tsegura@g.ucla.edu (T. Segura).

http://dx.doi.org/10.1016/j.biomaterials.2016.05.053
0142-9612/

slowing the gelation kinetics of the 8-arm and 4-arm PEG-maleimide (PEG-8Mal and PEG-4Mal) hydrogels to allow for better mixing of the macromers before percolation occurs. The microstructure was compared to the fully mixed 4-arm PEG-vinylsulfone (PEG-4VS) hydrogel, a slower Michael-type addition gelation, and 8-arm PEG-Norbornene (PEG-8N) step-growth polymerized hydrogel, which is completely homogeneous, since it can be fully mixed before initializing the reaction with light. Ultimately, the thiol containing peptide fragment off either end of the bi-functional-protease-cleavable degradable linker peptide (Ac-GXCXX-GPQGIWGQ-XXCXG-NH_2) (MMP_{XCXX}) was systematically optimized for the thiolate addition to the maleimide π-bond through the modulation of its pK_a.

2. Materials and methods

2.1. PEG hydrogel formation

The PEG-8Mal (40 kDa), PEG-4Mal (20 kDa), and PEG-4VS (20 kDa) macromers were obtained from JenKem Technology USA. PEG-8N was synthesized as previously reported [10]. PEG-8Mal, PEG-4Mal, and PEG-4VS hydrogels were formed in two steps. First, the thiol-containing adhesive peptide, GCGYGRGDSPG (RGD), was reacted with the maleimide or vinylsulfone functionalized PEG-8Mal, PEG-4Mal, or PEG-4VS at a concentration of 1 mM in 100 mM HEPES pH 7.4 for 10 min (0.3 M TEOA pH 8.2 for 30 min for PEG-4VS only) and then diluted in extra buffer [2]. Meanwhile, the di-thiol-containing linker peptide was reacted with 0.005 mM alexa fluor 350/555 maleimide (Life Technologies) to form MMP_{XCXX} solution. The solutions were combined with care, to maintain the final thiol to maleimide molar ratio of each gel condition and the final concentrations specified in Table S1. Throughout all the experiments the macromere solution, three-fifths of the final gel volume was pipetted between two sigma-coated glass slides, clamped together and separated by two 2 mm spacers, or into a 4 mm in diameter polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Kit, Ellsworth Adhesives) well plasma bonded onto a Corning microscope glass slide (Fisher Scientific). The MMP_{XCXX} solution consisting of two-fifths of the final gel volume was then mixed with the macromere solution, pipetting up-and-down until the solutions viscosity rendered the blow-out piston insufficient for pipetting the bulk solution. The injection, mixing and gelation was recorded with the Sandisk Extreme Pro camera (Bel Air Camera) over a UV lamp box.

2.2. pKa determination

The pKa of the MMP_{XCXX} thiol containing tag sequences (XCXX), Ac-GCRDG-NH¬2 (CRD), Ac-GDCDDG-NH2 (DCDD), Ac-GECEEG-NH2 (ECEE) (Genscript, Piscataway, NJ) was determined as previously described [11]. Briefly, 0.01 M sodium acetate, potassium phosphate, tris base, or sodium tetraborate decahydrate was added to 0.1 M NaCl to create various pH buffers. Aliquots with 0.15 μmol of lyophilized thiol-containing peptides, were dissolved in 1500 μL of pH buffers ranging from pH 4 to pH 12 for a final concentration of 0.1 mM. The absorbance at 240 nm of the thiolates in each aliquot was measured in a NanoDrop 2000c spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The absorbance of the thiolate was plotted versus pH and fitted to an adjusted Henderson-Hasselbalch equation where the pKa was indicated by the point of inflection of the graph.

2.3. Zinc IC50 assay

To analyze the toxic effects of zinc chloride on human dermal fibroblasts (HDF) the cell viability was evaluated according to the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay protocol. Briefly, 5000 cells per well were plated in a 96-well plate and 24 h later the media was exchanged with media containing 10 different concentrations of zinc chloride (obtained from even volume serial dilution of 16 mM zinc chloride down to 0 mM). Four hours later the media was exchanged for 190 μl fresh media and 20 μl of the combined MTS/PMS solution was added to each well. The plate was incubated for 4 h at 37 °C in a humidified, 5% CO_2 chamber and the absorbance at 490 nm was read using a plate reader and phase images were acquired using the Carl Zeiss fluorescent microscope with a 20× air objective. The absorbance readings were fit to an exponential decay line where the point of inflection represents the IC50 concentration of zinc chloride. All conditions were in triplicate.

2.4. Cell encapsulation

Human dermal fibroblasts (HDF) cells were encapsulated at a concentration of 3000 cells/μL into PEG-8Mal or PEG-8Norb hydrogels with 1 mM RGD, cross-linked MMP_{XCXX}*555 and cultured in DMEM + 5% fetal bovine serum + 1% penicillin/streptomycin. The media was refreshed every 3 days post-encapsulation and a set of gels were fixed at day 4, 7, 14, 21, and 28 with 4% paraformaldehyde (Fisher Scientific) for 30 min at room temperature after rinsing with 1xPBS. The hydrogels were stained with alexa fluor 488 phalloidin (Invitrogen) and DAPI or ToPro3 and then visualized on a Nikon-C1 laser scanning confocal microscope with a 20× air objective. Three-dimensional z-stack projection renderings are of a 100 μm thick portion of each gel.

3. Results and discussion

PEG-Mal hydrogels were formed through the rapid propagation of the thiolate onto the vinyl ring of the maleimide and the sequential chain-transfer of the hydrogen (Fig. 1A) [12–16]. To illustrate the bulk gel properties and the microdomain generation in Michael-type addition hydrogels, we used PEG-8Mal and PEG-4Mal macromere and the widely used degradable linker peptide (Ac-GCRD-GPQGIWGQ-DRCG-NH_2) (MMP_{CRD}). Gels were formed through the rapid mixing of the PEG-Mal macromere and MMP_{CRD} at pH of 7.4 in HEPES buffer. Visualization of the gel microstructure was achieved through using fluorescently labeled crosslinker (Alexa350) during gelation. Though bulk rheological properties (Fig. 1B) and visual inspection (Fig. 1C) of the entire 4 mm gel showed consistent mechanical properties and a homogeneous gel, visualization via confocal microscopy of the same 4 mm gel showed heterogeneous gelation (Fig. 1D). The hydrogels were highly heterogeneous containing pockets of uncrosslinked space or very loosely crosslinked space (low fluorescence space) surrounded by areas of highly crosslinked gel (highly fluorescent space, Fig. 1D). This is despite the fact that the PEG-Mal and MMP_{CRD} precursor solutions were carefully pH to 7.4 and mixed as quickly as possible. These low crosslinked pockets occurred at different locations and densities in a manner not predictable from experiment to experiment.

In order to investigate further how the regions of high and low crosslinking are generated, we recorded the gelation of the hydrogel. The PEG-Mal solution was first injected between two sigma-coated glass slides and the MMP_{CRD} solution was then added and pipetted back and forth for the best mixing of the two monomers (Fig. 1E and Video S1). Monitored for 10 s, the CRD hydrogel solutions became too viscous to mix any further by 4 s and 2 s leading to an uneven distribution of the MMP_{CRD} cross-linker as visualized by the zones of high fluorescence signal and low