Acta Biomaterialia 56 (2017) 80–90

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Bio-orthogonal conjugation and enzymatically triggered release of proteins within multi-layered hydrogels ☆

Chen Guo [a,1], Heejae Kim [a,1], Elisa M. Ovadia [a,1], Christine M. Mourafetis [b], Mingrui Yang [a], Wilfred Chen [a], April M. Kloxin [a,c,*]

[a] Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, United States
[b] Department of Chemical and Biomolecular Engineering, New York University, Brooklyn, NY 11201, United States
[c] Department of Materials Science and Engineering, University of Delaware, Newark, DE 19716, United States

ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form 31 March 2017
Accepted 3 April 2017
Available online 5 April 2017

Keywords:
Layered hydrogels
Bio-conjugation
Protein immobilization
Click chemistry
Protein release

ABSTRACT

Hydrogels are facile architectures for the controlled presentation of proteins with far-reaching applications, from fundamental biological studies in three-dimensional culture to new regenerative medicine and therapeutic delivery strategies. Here, we demonstrate a versatile approach for spatially-defined presentation of engineered proteins within hydrogels through *i)* immobilization using bio-orthogonal strain-promoted alkyne-azide click chemistry and *ii)* dynamic protease-driven protein release using exogenously applied enzyme. Model fluorescent proteins were expressed using nonsense codon replacement to incorporate azide-containing unnatural amino acids in a site-specific manner toward maintaining protein activity: here, cyan fluorescent protein (AzCFP), mCherry fluorescent protein (AzmCh), and mCh decorated with a thrombin cut-site. (AzTMBmCh). Eight-arm poly(ethylene glycol) (PEG) was modified with dibenzylcyclooctyne (DBCO) groups and reacted with azide functionalized PEG in aqueous solution for rapid formation of hydrogels. Azide functionalized full-length fluorescent proteins were successfully incorporated into the hydrogel network by reaction with PEG-DBCO prior to gel formation. Temporal release and removal of select proteins (AzTMBmCh) was triggered with the application of thrombin and monitored in real-time with confocal microscopy, providing a responsive handle for controlling matrix properties. Hydrogels with regions of different protein compositions were created using a layering technique with thicknesses of hundreds of micrometers, affording opportunities for the creation of complex geometries on size scales relevant for controlling cellular microenvironments.

Statement of Significance

Controlling protein presentation within biomaterials is important for modulating interactions with biological systems. For example, native tissues are composed of subunits with different matrix compositions (proteins, stiffness) that dynamically interact with cells, influencing function and fate. Toward mimicking such temporally-regulated and spatially-defined microenvironments, we utilize bio-orthogonal click chemistry and protein engineering to create hydrogels with distinct regions of proteins and modify them over time. Through nonsense codon replacement, we site-specifically functionalize large proteins with *i)* azides for covalent conjugation and *ii)* an enzymatic cleavage site for user-defined release from hydrogels. Our results exemplify not only the ability to create unique bio-functionalized hydrogels with controlled mechanical properties, but also the potential for creating interesting interfaces for cell culture and tissue engineering applications.

1. Introduction

Synthetic hydrogels are excellent modular platforms for use as sensors [1], drug delivery vehicles [2], and biomimetic scaffolds for tissue engineering applications [3,4]. Synthetic polymers such as poly(ethylene glycol) (PEG) are easily functionalized with a variety

☆ Part of the Gradients in Biomaterials Special Issue, edited by Professors Brendan Harley and Helen Lu.

* Corresponding author at: 150 Academy Street, Colburn Laboratory 207, Newark, DE 19716, United States.
E-mail address: akloxin@udel.edu (A.M. Kloxin).

[1] These authors noted with equal contribution.

http://dx.doi.org/10.1016/j.actbio.2017.04.002
1742-7061/

of reactive functionalities to provide a bioinert base for the creation of well-defined network architectures with high water content and controlled physical and biochemical properties [5]. In these materials, the incorporation and removal of biomolecules, such as proteins, is key for directing interactions with biological systems, including mammalian cells in culture and in the body for modulating their attachment, function, and fate [3]. A simple approach for protein incorporation into hydrogels is encapsulation and entrapment of full-length proteins; while effective for therapeutic delivery applications, use in cell culture typically is limited to only large proteins (e.g., whole laminins, fibronectin) that can be retained in select hydrogel compositions [6]. For more precise control of protein presentation from and within hydrogels, covalent modification of proteins can be broadly useful.

A variety of approaches with different levels of complexity have been developed for protein immobilization. These include *i)* orthogonal physical binding pairs (e.g., barnase-barstart and streptavidin-biotin) [7], *ii)* oxime ligation [8], and *iii)* thiol-ene reaction [9], where photo-uncaging or photo-initiating chemistries have been applied for spatial specificity of immobilization. While useful, these methods often rely on chemical modification of proteins by reacting with randomly-located amine or thiol groups on the protein backbone. Consequently, these modifications typically are not site-specific and the number of modifications on each protein cannot be easily controlled, which may cause potential undesired side reactions such as disulfide bond formation between proteins. In addition to these undesired side reactions, the modification can occur at an active site of the protein, with the potential to cause protein misfolding or dysfunction.

A few methods have emerged for enzyme-mediated covalent coupling of engineered proteins and protein fragments to preformed hydrogels with sortase A [10] and transglutaminase factor XIIIa (FXIIIa) [11], respectively. Additionally, single lysines have been incorporated within protein sequences designed for assembly to enable the subsequent introduction of reactive functionalities and covalent modification for crosslinking [12]. These approaches demonstrate the power of genetic modification for maintaining protein bioactivity during covalent coupling and immobilization. However, challenges remain in diffusion of enzymes and proteins into gels for homogenous reaction and in site-specific protein modification with reactive unnatural amino acids (UAAs) for maximizing coupling efficiency. Versatile methods that enable site-specific modification of proteins and bioorthogonal conjugation and immobilization are needed for the design of biomimetic materials with well-defined cell-matrix interactions.

The ability to control immobilization and removal of proteins from the hydrogel is of growing interest for a number of biological applications [8]. In the human body, gradients of soluble proteins, such as morphogens, growth factors, and cytokines, occurring over hundreds of micrometers at specific times are known to drive tissue morphogenesis in development and regeneration in wound healing [13,14]. For example, gradients of chemokines and matrix stiffness drive stem cell migration and condensation during cartilage development, ultimately leading to a shift from a fibronectin-rich to collagen-rich environment that facilitates stem cell differentiation and tissue development [15,16]. Materials that enable the release of proteins in time, as well as an anisotropic manner, are needed for studying and directing these processes within culture systems and implanted devices. One method to achieve this is through the cleavage and subsequent release of covalently-linked proteins. Cleavage of tethered proteins from hydrogel-based matrices has been accomplished through incorporation of photolabile groups [8,17] or protease cleavage sites [18]. For example, incorporation of an *o*-nitrobenzyl group has been used to spatiotemporally control cleavage of integrin-binding proteins upon irradiation for directing MSC differentiation before and after protein cleavage [8].

To better mimic anisotropic, multipart tissues, patterned and more complex structures are required, such as intricate hydrogels containing multiple layers with different biochemical cues, various mechanical properties [19], or multiple cell types [20]. For example, to mimic the structure of cartilage which is composed of different zones, a multi-layer hydrogel composite was formed with different mechanical and biochemical properties within each layer to promote zone-specific chondrogenic differentiation of MSCs [19]. Additionally, co-culture of neural progenitor cells, astrocytes, and neurons each within a different layer of a multi-layer hydrogel accelerated neural migration and differentiation [20]. Methods that combine cleavage strategies with such layering techniques would provide a straightforward approach to controlling the presentation of proteins at times and locations of interest and facilitate bottom up design over multiple size scales, from the molecular to micro- and macro-scales.

Genetic engineering is a powerful tool for introducing unique and site-specific modifications to proteins that enable orthogonal coupling and cleavage reactions. Site-specific incorporation of UAAs for functionalizing proteins can be achieved using nonsense codon replacement [21–24]. Unlike sense codon replacement [25,26], nonsense codon replacement allows for the site-specific placement of UAAs in each protein molecule using either a stop codon or 4-base codon as the designator for the UAA. This strategy for placement of the UAA also minimizes protein dysfunction when, by design, the conjugation site is away from the active site of the protein. Furthermore, UAAs can be strategically placed alongside specific domains, such as protease cleavage sites, giving the benefits of unparalleled site-specific chemical conjugation and a specific biological release mechanism for the construction of well-defined and dynamic biomaterials.

Here, we report a modular approach for the controlled incorporation and removal of proteins in hydrogels using a combination of bio-orthogonal click chemistry and protease-responsive engineered proteins within defined geometries. Multi-arm PEG was functionalized with dibenzocyclooctyne (DBCO), which undergoes biocompatible and biorthogonal strain promoted alkyne-azide cycloaddition (SPAAC) click chemistry [27,28] with a di-azide functionalized PEG (PEG-2-Az) crosslinker for gel formation. The UAA p-azido-l-phenylalanine (pAzF), which provides azide functionality, was site-specifically incorporated into a cyan fluorescent protein (CFP), a mCherry fluorescent protein (mCh), and mCh decorated with a thrombin cut-site (AzTMBmCh). Gels containing layers with different protein compositions were created using a layering methodology, and in combination with the engineered proteins, permitted spatially-defined protein presentation initially and in time upon thrombin application. Immobilization of full-length proteins within hydrogels followed by controlled temporal enzymatic cleavage in defined regions is promising for use in a number of biological applications, including controlled cell culture, tissue engineering, diagnostics, and therapeutic delivery.

2. Materials and methods

Eight-arm amine-functionalized poly(ethylene glycol) (PEG-8-NH_2, 40 kDa) was purchased from JenKem Technology (Beijing, China). Bovine Serum Albumin (BSA) and dibenzocyclooctyne-acid (DBCO-Acid) were purchased from Sigma Aldrich (St. Louis, MO). HATU was purchased from ChemPep (Wellington, FL). N-N-Dimethylformamide (DMF, 99.8% anhydrous) was purchased from Acros Organics. PEG-*bis*-azide (PEG-2-Az, 3.4 kDa) was purchased from Creative PEGWorks. Dulbecco's Phosphate Buffered Saline (DPBS, 1X) was purchased from Thermo Fisher Scientific (Grand