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Mimicking the extracellular matrix with functionalized, metal-assembled collagen peptide scaffolds

Victor Hernandez-Gordillo, Jean Chmielewski*

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907, USA

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ABSTRACT

Natural and synthetic three-dimensional (3-D) scaffolds that mimic the microenvironment of the extracellular matrix (ECM), with growth factor storage/release and the display of cell adhesion signals, offer numerous advantages for regenerative medicine and *in vitro* morphogenesis and oncogenesis modeling. Here we report the design of collagen mimetic peptides (CMPs) that assemble into a highly crosslinked 3-D matrix in response to metal ion stimuli, that may be functionalized with His-tagged cargoes, such as green fluorescent protein (GFP-His_8) and human epidermal growth factor (hEGF-His_6). The bound hEGF-His_6 was found to gradually release from the matrix *in vitro* and induce cell proliferation in the EGF-dependent cell line MCF10A. The additional incorporation of a cell adhesion sequence (RGDS) at the N-terminus of the CMP creates an environment that facilitated the organization of matrix-encapsulated MCF10A cells into spheroid structures, thus mimicking the ECM environment.

1. Introduction

The extracellular matrix (ECM) is a complex mixture of structural proteins (collagen, laminin, and fibronectin), polysaccharides (glycosaminoglycans) and signaling proteins such as growth factors (GFs) [1]. Together, the components of the ECM offer the physical and biochemical cues for cells to attach, grow, proliferate, migrate and differentiate [2,3]. Three-dimensional (3-D) scaffolds that can mimic some features of the ECM in terms of cell growth, organization and differentiation are of pivotal importance in areas such as regenerative medicine, and morphogenesis and oncogenesis modeling *in vitro* [3,4].

Cell adhesion motifs present in the structural proteins of the ECM are key components in various types of cell–cell, cell-ECM, and cell-soluble molecule (such as GFs) interactions [5]. Collagen, fibronectin and vitronectin, among other proteins in the ECM contain the RGD (Arg-Gly-Asp) cell adhesion motif [6]. Over half of the known integrin-binding proteins in cells recognize and bind this sequence, thus RGD and its derivatives (e.g. RGDS, YRGDS) are the most common cell adhesion motifs covalently incorporated into 3-D scaffolds lacking cell adhesion sequences [6]. This is accomplished by chemically linking the cell adhesion sequence to functional groups (hydroxyl-, amino-, or carboxyl groups) present in the synthetic or natural scaffolds [7–9]. As a result, cells cultured on RGD-modified scaffolds often have better cell attachment, spreading, and differentiation than the unmodified counterparts [7,10,11].

GFs, another important component of the ECM, are anchored on cells or trapped within the ECM *in vivo*. Signaling can occur with both anchored and soluble forms of GFs [2]. It is advantageous, therefore, for an ECM-mimicking scaffold to supply the GFs within the matrix itself. However, immobilization of GFs onto 3-D matrices is challenging and is currently an area of extensive research [12–15]. GFs can be immobilized through covalent and non-covalent interactions depending on the properties of the material. For non-covalent immobilization, unmodified GFs can be physically entrapped, adsorbed, or complexed with the scaffold [16–18], or alternatively GFs can be modified with a collagen-binding domain to promote scaffold interactions [19]. For covalent modification, the GFs can be linked to an activated scaffold via functional groups already present in the material, such as amines [16,20]. GFs can also be functionalized with activated esters [21,22], photo-reactive groups (e.g. phenyl azide) [23,24] and cysteine tags [25,26] in order to react with functional groups present in the scaffold. The addition of sulfhydryl groups to both the scaffold and GF can also promote crosslinking and the covalent incorporation of GFs [27]. The immobilization of GFs onto scaffolds has several benefits, such as reduced GF degradation, prolonged bioactivity [15] and stronger

* Corresponding author. Fax: +1 765 494 0239.
E-mail address: chml@purdue.edu (J. Chmielewski).

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signaling potency compared to their soluble counterparts [28,29]. In addition, GF immobilization is preferable for tissue regeneration applications as compared to intravenous systemic delivery, as the latter often results in the accumulation of GFs at other sites of the body besides the implantation site, or to short-lived biological activity due to degradation [30,31].

Significant progress has been made in the design of 3-D scaffolds for tissue engineering applications. Scaffolds based on natural polymers, such as collagen and fibrin, offer a number of benefits like presenting cells with cell adhesion sequences found in the ECM. However, such scaffolds can trigger immunogenic reactions when implanted in humans and difficulties with batch-to-batch variability may affect the results of *in vitro* and *in vivo* assays [32–34]. Also, these materials are difficult to modify with additional functionality, such as non-native cell adhesion sequences for tailored applications. Synthetic scaffolds based on polylactic acid (PLA), polyglycolic acid (PGA) and polyethylene glycol (PEG) are reasonable alternatives to natural scaffolds as they may mimic the mechanical properties (e.g. stiffness) of the ECM and can be functionalized with cell adhesion sequences and GFs [9,35,36]. However, their fabrication and modification requires multiple steps and conditions (e.g. UV, organic solvents) that could diminish the bioactivity of the GFs or the viability of cells being encapsulated [37]. Consequently, cells are usually added on the surface after scaffold assembling, rendering the material as a two-dimensional scaffold. Thus, there is a demand for bioscaffolds that can incorporate GFs in combination with cells within the scaffold prior to implantation [13,38].

A new set of scaffolds, based on peptide mimetics has strong potential for use in the field of tissue engineering, because they combine the functionality of protein-based biomaterials with the reproducibility of synthetic scaffolds [39–41]. These peptide mimetics are generally short sequences (15–45 residues), based on the amino acid sequences of naturally occurring proteins, although various *de novo* peptide sequences also exist [42–45]. For instance, collagen mimetic peptides (CMPs) are of interest because they emulate some of the features of natural collagen [46]. CMPs with a $(Pro\text{-}Hyp\text{-}Gly)_n$, (POG) sequence form stable triple helices that are similar to the triple helices found in natural collagen [46].

CMPs have been shown to assemble into higher order structures beyond the triple helix using a variety of strategies [40,47,48]. For instance, an unmodified $(POG)_{10}$ peptide sequence self-assembled into nanoscale branched structures after thermal annealing [49]. Nano- and micro-meter size fibers were obtained by polycondensation of $(POG)_{10}$ or by native chemical ligation of $(POG)_9$ peptides [50,51]. Cysteine knots within CMPs containing staggered overhangs have also been used for self-assembly, resulting in particles in the range of 0.6–14 μm size, fibers of >400 nm length, nanorods, and micro-size fibrils [52–54]. The addition of an Arg residue to the C-terminus of the CMPs, in combination with the cysteine knot, resulted in the formation of collagen-like gels [55,56].

Modifications at the N- and C-terminus of CMPs have been shown to influence higher order assembly. For instance, placing aromatic residues at the termini of CMPs promoted hydrophobic interactions between triple helical end groups that resulted in the formation of micron-sized fibers [57–59]. Similarly, the addition of a cationic residue at the N-terminus and an aromatic residue to the C-terminus was found to promote a head-to-tail assembly through cationic-π interactions resulting in micrometer size fibrils [60]. Placement of Arg and Glu residues at the N- and C-terminus of CMPs promoted head-to-tail electrostatic interactions, as well as lateral assembly, that resulted in fibers with a periodic banding pattern similar to natural collagen [61]. Lys and Asp residues at the termini resulted in hierarchical assembly of fibers to hydrogels within a few hours [62]. Adding single-tail amphiphilic moieties to the N-terminus of CMPs stabilized the CMPs helices and promoted the formation of fibers and collagen-like hydrogel [63,64].

An alternative strategy for CMPs assembly has been the use of metal-ligand interactions. By placing ligands for metal ions at different locations within CMPs, metal-promoted higher order assembly has been achieved. This strategy has provided a wide variety of structures, including nano- and micro-sized fibers [65,66] disks [67], hollow spheres [68], florettes [69–72], and 3-D matrices [71,73]. The presence of unsatisfied metal-ligand interactions at the surface and within the scaffold, allowed the incorporation of His-tagged biomolecules [70]. Additionally, the simultaneous presentation of ligands at the termini and center of a CMP facilitated the metal-ion promoted assembly of a 3-D scaffold under physiological conditions (*i.e.* buffered aqueous solutions, cell culture media with serum), allowing for facile encapsulation of cells [73].

Overall, CMPs can self-assemble into fibers and hydrogels that resemble features of natural collagen. However, these materials have shown limited use in applications for tissue engineering. In the present study, we investigated the feasibility of using the 3-D matrix-forming CMP, **HBN** (Fig. 1), for GF delivery and as a scaffold for cell growth and organization. To further mimic the ECM [74], we incorporated the cell adhesion sequence Arg-Gly-Asp-Ser (RGDS) at two different positions within the **HBN** peptide, resulting in two *de novo* peptide sequences, **HRGDSN** and **HBRGDS** (Fig. 1). Upon triple helix formation, these peptides would present the RGDS sequences to cells as single strands radiating out from the center of the triple helix (**HRGDSN**) or as trimeric bundles at the N-terminus of the triple helix (**HBRGDS**). In our design, GFs and cells would be incorporated within the 3-D matrix under physiological conditions during scaffold assembly, so as to maintain the bioactivity of the GF and the viability of the cells.

Herein, we investigate if a human non-tumorigenic epithelial cell line (MCF10A) that is encapsulated in a 3-D matrix based on the **HBN** peptide will respond to the designed scaffold and form cellular spheroids. Spheroids of MCF10A cells have been formed using the natural Matrigel™ scaffold that contains more than 1800 proteins, including collagen, laminin, fibronectin and numerous GFs [75]. Thus, we hypothesized that the designed minimalistic scaffold of **HBN** would function as an ECM-like environment for MCF10A cell growth and organization *in vitro* due to the incorporation of epidermal growth factor (EGF) and the RGDS cell adhesion sequence within the CMP matrix.

2. Material and methods

2.1. Materials

The Rink Amide ChemMatrix for solid phase peptide synthesis was purchased from Pcas-Biomatrix Inc (Quebec, Canada). Fmoc-protected amino acids, and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluraniumhexafluorophosphate (HBTU) were purchased from AAPPTec (Louisville, KY, USA), ChemPep Inc (Wellington, FL, USA) and Chem-Impex International (Wood Dale, IL, USA). Piperidine and diisopropylethylamine (DIEA) were purchased from Alfa Aesar (Ward Hill, MA, USA). Acetonitrile, triisopropylsilane (TIPS), trifluoroacetic acid (TFA), *N*-*N*-dimethyl formamide (DMF), methanol, Tween 20, poly(2-hydroxyethyl methacrylate (pHEMA) and nickel(II) chloride hexahydrate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dichloromethane (DCM) was purchased from AVANTOR (Center Valley, PA, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA) was purchased from Mallinckrodt JT Baker (Hazelwood, MO, USA).

The His-tagged green fluorescence protein ($GFP\text{-}His_8$) was obtained as a gift from Christine Hrycyna (Purdue University). His-tagged human epidermal growth factor ($hEGF\text{-}His_6$) was purchased from Aviscera Bioscience (Santa Clara, CA, USA). Rabbit anti-human EGF (500-P45) was purchased from PeproTech (Rocky Hill, NJ, USA). Mouse anti-GFP clone 3E6 (A-11120) was purchased from Life Technologies (Grand Island, NY, USA). Mouse anti-His tag antibody (A00613) and streptavidin-horseradish peroxidase (HRP, M00091) were purchased from GenScript (Piscataway, NJ, USA). The TMB (3,3′,5,5′-tetramethylbenzidine) kit (34021) was purchased from Thermo Scientific. Calcein AM was purchased from Biotium (Hayward, CA, USA). Hoechst 33342 was purchased from AnaSpec (Fremont, CA, USA). Bovine serum albumin (BSA), cholera toxin, and hydrocortisone were purchased from Sigma–Aldrich (St. Louis, MO, USA). The MTS colorimetric assay was purchased from

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