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Hemostatic properties and the role of cell receptor recognition in human hair keratin protein hydrogels

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ABSTRACT

Driven by new discoveries in stem-cell biology and regenerative medicine, there is broad interest in biomaterials that go beyond basic interactions with cells and tissues to actively direct and sustain cellular behavior. Keratin biomaterials have the potential to achieve these goals but have been inadequately described in terms of composition, structure, and cell-instructive characteristics. In this manuscript we describe and characterize a keratin-based biomaterial, demonstrate self-assembly of cross-linked hydrogels, investigate a cell-specific interaction that is dependent on the hydrogel structure and mediated by specific biomaterial–receptor interactions, and show one potential medical application that relies on receptor binding - the ability to achieve hemostasis in a lethal liver injury model. Keratin biomaterials represent a significant advance in biotechnology as they combine the compatibility of natural materials with the chemical flexibility of synthetic materials. These characteristics allow for a system that can be formulated into several varieties of cell-instructive biomaterials with potential uses in tissue engineering, regenerative medicine, drug and cell delivery, and trauma.

1. Introduction

Biomaterials have historically been used in applications where their function is primarily structural and inert, a characteristic that has largely been emphasized over biological interaction. Applications for medical devices and drug delivery have benefited greatly from this approach, but more recently the sophistication of biomaterials has increased dramatically and new, information rich materials are being developed, primarily by inspiration from nature. These new biomaterials have broad application and their use as replacements for biological tissues, devices for trauma and surgical applications, and in medical diagnostics have fueled a renaissance in biomaterials science [1].

Many innovative medical applications employ the engineering of several aspects of biologically relevant behavior into novel biomaterials. Key among these characteristics are adhesion of cells, promotion of cell growth and/or differentiation, and in the case of tissue engineering, appropriate structure and cell-mediated degradation. New insights have shown that physiologically relevant adhesion of the target cells is an essential first step in most tissue engineering applications as important processes such as migration and outside-in signaling are influenced by specific receptor recognition of the biomaterial surface [2]. Next, growth and differentiation of seeded exogenous or infiltrating endogenous cells must be facilitated as tissue morphogenesis ensues. Pharmacological control through the use of growth factors can direct some of these behaviors [3]. Beyond gross anatomical structure, biomaterial assembly from the nanometer to micron scales has important implications to how cells recognize, organize and respond to the temporary extracellular environments presented by biomaterial implants [4,5]. Lastly, the goal of most of these systems is to serve as a temporary matrix for tissue regeneration. Therefore, cell-mediated degradation is also an important consideration so that it can be synergistically coordinated with new tissue formation. [6]

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Such biomaterial systems are the focus of intense development based largely on the fact that many materials with a history of safe use in humans lack the ability to be engineered to the specifications required by the leading edge medical applications contemplated by many scientists. Potential solutions have been presented in the form of synthetic polymers that contain binding motifs to facilitate cell adhesion [7], growth factor presentation to promote chemotaxis, cell division and/or differentiation [8], physical features that can direct cell behavior [9], enzymatically degradable crosslinks [10], and combinations thereof. Alternatively, some investigators have taken the approach of modifying natural extracellular matrix (ECM) biopolymers that have inherent biological properties to impart the desirable characteristics of synthetic polymers such as processability and controlled degradation rates [11,12]. Keratins fall into this later category of biopolymers but do not require the same chemical modification to impart usefulness in terms of tunable physical and degradation characteristics. Despite these obvious advantages, keratins have not been extensively studied as a biomaterial system compared to other structural proteins.

Keratin refers to a broad category of insoluble proteins that associate as intermediate filaments in either epidermal appendageal structures (e.g. hair, wool, horns, hooves and nails) [13] or in bundles of cytoplasmic filaments that provide mechanical resilience to epithelial cells [14]. The structural subunits of both epithelial and trichocytic (i.e. hair) keratins are two chains of differing molecular weights and amino acid compositions that each contain non-helical end-terminal domains and a highly-conserved, central alpha-helical domain. Human hair keratins are similar in composition to the ubiquitous epithelial keratins but differ in that they contain a relatively high percentage of cystine residues in their non-helical domains [15,16]. Keratins exist as obligate heterodimers that self-assemble into intermediate filaments, which further self-assemble to form higher ordered nano-structures that make up the keratinous tissues of vertebrates (e.g. hair, wool, nails, feathers, etc.).

The cystine residues in disulfide crosslinked keratinous tissues can be converted through reductive chemistry to cysteines. Trichocytic keratin proteins can be extracted from end-cut human hair fibers by a variety of chemical techniques that have been widely published [17]. Intra-molecular crosslinks must first be broken down so that cortical keratins can be made soluble and extracted, but in a way that does not damage their molecular integrity. After purification, typically by isoelectric precipitation and/or dialysis, the resulting extract represents a complex mixture of type I and II alpha keratin dimers [18], keratin-associated proteins (KAP) derived from the matrix of the hair cortex [19], and fragments of both that are often referred to in the literature as “gamma” keratins [13]. Polymerized (i.e. entangled and disulfide crosslinked) keratin biomaterials can be formed from these molecules, with more emphasis in most publications placed on description of the extraction and purification processes than on protein identification.

While many useful properties of keratin biomaterials have been described [17], a more revealing examination of their composition and molecular structure, as well as the influence that these characteristics have on cell–biomaterial interactions, is essential to facilitating their widespread adoption in biotechnology applications. A framework for understanding these structure-property relationships has been made possible through a description of their primary [18], and to a more limited extent, secondary and tertiary structures [16], as well as recent structure-property relationship studies of specific keratin systems [20,21]. Moreover, insights into hair keratin self-assembly [22,23] have added to this framework and set the stage for a more thorough investigation of the interactions of keratin biomaterials with cells.

Keratin biomaterials can be formulated to have a long persistence time *in vivo* due their ability to form biologically stable disulfide crosslinks, as well as the absence of specific keratinases in mammals. Thus, keratins are not susceptible to rapid proteolytic degradation as is common with other biologically derived materials such as collagen. Keratin biomaterials are well-tolerated in living systems, and have been tested in a variety of animal models that simulate several potential biomedical applications [24–29], while other studies have shown keratins to be capable of supporting cellular attachment [30–35], purportedly through binding motifs such as leucine-aspartic acid-valine (LDV) and glutamic acid-aspartic acid-serine (EDS) peptide domains present in the type I alpha keratins [31,33]. Notably, mechanism(s) of action in these studies has gone largely unexplored. Here we describe a keratin biomaterial system composed of well-characterized proteins, show their self-assembly into crosslinked porous hydrogels, investigate the cell–biomaterial interactions that may be responsible for a particular cell adhesion mechanism, and demonstrate an important medical application for this unique biomaterial system.

2. Methods

2.1. Keratin biomaterial processing

The methods to extract keratin proteins from human hair have been described previously [20]. Briefly, a cold solution of 1 M thioglycolic acid (TGA) in NaOH is added to 250 g of untreated virgin Asian hair (World Response Group Inc. Florida City, FL) and placed on a shaker overnight for 12 h. TGA at basic pH serves to break disulfide bonds that hold both the cuticular and cortical structures together, and allows extraction of soluble proteins from the hair fiber. The solution was then passed through a 500 micron sieve and the hair collected. A 40-fold excess of 100 mM tris base was added to the reduced hair fibers and the container shaken vigorously at 37 °C for 2 h to extract additional soluble proteins from the hair fiber. The solution was removed using the same sieve and a 40 fold excess of DI water was added to the hair, which was shaken again at 37 °C for 2 h to extract any remaining soluble proteins from the hair fiber. All solutions were combined and spun on a centrifuge at 6000 rpm for 40 min then filtered using Whatman #4 filter paper. The alpha and gamma keratins were then separated by acid precipitation using dilute HCl and dialyzed in a custom-made dialysis system using a 3 kDa nominal low molecular weight cutoff tangential flow spiral wound filter for the gamma fraction (Prep/Scale TFF spiral wound ultrafiltration module; Millipore, Billerica, MA) and 30 kDa molecular weight cutoff filter for the alpha fraction (Millipore). The alpha and gamma fractions were then recombined at a ratio of 98% alpha and 2% gamma, neutralized to pH 7.4, and concentrated using a rotoevaporator to reach a 12% keratin solution. 10 cc of this solution was drawn into a syringe and placed overnight in a 37 °C room to cross-link and form a gel. The syringes were then gamma sterilized at a dose of 10 kGy, making them shelf stable and able to be stored long term. Powdered keratin samples were prepared by lyophilizing the 12% keratin solution, grinding and sterilizing the dry protein.

2.2. Size exclusion chromatography

In order to assess the native molecular weight of the keratin compounds isolated by the process described above, size exclusion chromatography (SEC) was performed. Samples were dissolved at 1–2 weight/volume percent (depending on viscosity) in 10 mM HEPES + 130 mM sodium chloride, pH 7.4 buffer. A Biologic LP chromatography system (Bio-Rad Laboratories, Hercules CA) using a 1 × 30 cm Sephacryl S-300 column (GE Healthcare, Piscataway, NJ) running at 0.17 mL/min was injected with approximately 800 μL of sample. Detection of proteins was at 250 nm. Standards at 340, 230, 128, and 64 kDa molecular weight were analyzed under identical conditions and molecular weights of the keratin samples calculated from the Log(10) molecular weight-peak elution time relationship.

2.3. Electrophoresis and mass spectrometry

To separate and visualize the keratin proteins, isoelectric focusing (IEF) and 2-D gel electrophoresis were performed. Keratin (250 μg) was precipitated with 5% trichloroacetic acid (TCA), washed with acetone and air dried. The pellet was then dissolved in 200 μL of ReadyPrep™ rehydration buffer (Bio-Rad, Hercules, CA) and loaded onto immobilized pH gradient (IPG) strips (pH 3–10; Bio-Rad). Following overnight rehydration, the strips were focused using a three step protocol (250 V for 20 min; 8000 V for 2.5 h; 8000 V at 20,000 V-h) and then equilibrated for electrophoresis. Bio-Rad Criterion Tris–HCl gels (8–16%) were used for 2-D electrophoresis, which was run at a constant voltage of 200 V for about 65 min. Gels were stained with commassie blue and destained in methanol/acetic acid. Protein spots were isolated and submitted to the Wake Forest University Bioanalytical Core Laboratory for identification by mass spectrometry (MS).