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Molecular mechanism and characterization of self-assembly of feather keratin gelation

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ABSTRACT

Protein gels with controlled viscoelastic properties could find numerous material and biomedical applications. Feather keratin is naturally abundant protein while its gelation property has not been explored. In this study hydrogel from fully reduced feather keratin was prepared by dialysis. The objectives of this work were to study the molecular mechanism of self-assembly of feather keratin gel and to characterize the structural and viscoelastic properties of hydrogels prepared under various pHs (3–9) and temperatures (50–90 °C). Re-oxidation of free cysteine thiols and formation of hydrophobic interactions and hydrogen bond were determined as the main stabilizing forces in self-assembly of feather keratin gel. Adding thiol blocking agent of *N*-ethylmaleimide leads to reduced storage modulus of keratin gel; gelation was completely inhibited at 82% blockage of free thiols. Increasing temperature decreased storage modulus, while gelation at pH 3 resulted in stiffer gels compared to pHs of 5, 7 and 9. Feather keratin gels with tunable viscoelastic properties could find applications as engineered scaffolds for different tissues.

1. Introduction

Gel scaffolds prepared from proteins are of particular interest due to their abundance, biodegradability, compatibility with biological systems and the presence of peptide motif important for cell adhesion [1,2]. Keratins are fibrous proteins widely found in animal tissues including hair, skin epidermis, wool, hooves, scales and feathers. Keratin gel scaffolds from hair and wool has demonstrated good biocompatibility for tissue engineering applications [3–6]. However, there is scanty information on feather derived keratin gels in literature.

Keratins from chicken feather are an abundant yet underutilized protein source. As a result of poultry processing, over 65 million tons of feathers are generated worldwide [7]. Keratin accounts for more than 90% (w/w) of feathers. A remarkable feature of keratins is their high content of cysteine residues (7–20 mol%) in disulfide bonds, which confer strength and impermeability to keratinous tissues [6]. Solubilization of keratin is a limiting factor for the development of films, coatings and gels [8]. Thiols, sulfites, and urea are often used to solubilize keratins [9]. However, aggregates formed after removal of the denaturing chemicals are considered as an undesirable phenomena from the processing point of view [10,11]. The aggregation resultant from dialysis or dilution of reduced feather keratin solution was reported early [12]. More recently it was found that the reformation of disulfide bonds during dialysis of feather keratin was the main factor for the formation of aggregates [13]. Therefore, blocking of cysteine thiols has been applied to solublilize keratins, either using chemical grafting [14,15], oxidation into sulfonic acids [16], or by the formation of keratin sodium dodecyl sulfate (SDS) complexes [8,17,18]. Suprisingly, the spontaneous aggregation of fully reduced keratin has not been taken as an advantage for the fabrication of strong gels.

Hydrogels from fully reduced wool and hair keratin have superior mechanical properties [5,19,20]. Feather keratins differ from wool and hair since they have lower molecular weight, and are predominant in β-sheet secondary conformation [21]. It has been recognized that disulfide bonds play a major role in the formation of small aggregates in feather keratin solutions [13,22]. However, it has not been determined at which extent disulfide reformation or other intermolecular interactions are necessary for the formation and modulation of viscoelastic properties of feather keratin gels. Viscoelastic and physical properties of hydrogel scaffolds can affect different aspects of cellular behavior including, cell adhesion, morphology, survival and tissue regeneration [23]. Therefore, the determination of conditions and mechanisms that control the gelation of fully reduced feather keratin could help to modulate physical and biological properties of hydrogels.

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2. Experimental section

2.1. Materials and chemicals

White chicken feathers were obtained from the Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). After exhaustive washing with hot water and domestic soap (Green Works®), feathers were allowed to dry in a fume hood followed by drying at 50 °C overnight in an oven. Dried feathers were ground in a cutting mill (Fritsch Pulverisette 15, Fritsch, Germany) with an insert sieve of circular perforations of 1 mm diameter. Ground feathers were defatted with petroleum ether for 5 h in a soxhlet apparatus. The defatted feathers were recovered and the residual solvent was allowed to evaporate overnight at room temperature. Defatted feather powder was stored at 4 °C for further uses. Urea, thiourea, sodium metabisulfite, *N*-ethyl maleimide (NEM), 5,5′-Dithiobis(2-nitrobenzoic acid) (DNTB; Ellman's reagent), hydrogen peroxide, 1,4-dithiothreitol (DTT), formaldehyde solution 36.5-38%, and Sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (St Louis, MO, U.S.).

2.2. Preparation of feather keratin hydrogels

Extraction of keratin was performed according to Nakamura et al., [9] with modifications. Briefly 13.3 g of defatted feather powder was mixed with 200 mL of extracting solution consisting of 5 M urea, 2.6 M thiourea and 5% w/v sodium metabisulfite at pH 7.0, 50 °C for 5 h. Non-solubilized feather powder was removed by filtration through a metallic sieve (53 μm) and the filtrate was used for gelation experiments. The protein concentration of the gelling solution was calculated to 4.20 ± 0.20 wt/vol%, measured by Dumas's method of freeze dried gels in a Leco Truspec C/N Analyzer (Leco Corporation, MI, USA). Gelation of keratin samples was induced by dialysis using a molecular weight cut-off of 3500 Da for 5 days at 4 °C with change of the outer distilled water twice per day.

2.3. Effects of cysteine thiols on gelation

To study the effect of cysteine thiol groups on gelation, keratin solution recovered by filtration after extraction was reacted with various *N*-ethylmaleimide (NEM)/cysteine molar ratios (1.0, 2.5, 5.0 and 10.0; namely NEM 1, NEM 2.5, NEM 5.0 and NEM 10, respectively) in order to prevent disulfide bond re-formation (See Supplementary material for calculation of NEM/cysteine molar ratio). The pH was maintained at 7 by adding continuously 3 M HCl when needed and the solution was stirred at room temperature for 1 h. Gelation was induced by dialysis for 5 days at 4 °C with change of the outer water twice per day.

2.4. Effect of pH and temperature on gelation

Keratin solutions were adjusted to pH 3, 5, 7, and 9 (at room temperature) and stirred for 1 h before inducing gelation by dialysis as described before. To study the effect of temperature, keratin solutions were heated at 50, 60, 70, 80, and 90 °C (pH 7) in a water bath for 1 h. Gelation was induced by dialysis and soluble protein after gelation recovered for soluble protein quantification determined using a Lowry protein assay kit (Thermo Scientific, USA) using bovine serum albumin as the standard and SDS-PAGE under reducing conditions.

2.5. Time of flight-secondary ion mass spectrometry (TOF-SIMS)

Ion mass spectra of keratin and *N*-ethylmaleimide (NEM) modified keratin powder pellets were recorded using a TOF-SIMS imaging spectrometer (ION-TOF GmbH, Münster, Germany), equipped with a dual beam profiling (Ar/O2/Cs for layer removal and Ga for analysis).

2.6. Fourier transform infrared spectroscopy (FTIR)

Fourier transforms infrared (FTIR) spectroscopy analysis of keratin samples was performed in a Nicolet iS50 FTIR spectrometer (Thermo Scientific Inc., WI, USA) equipped with a single bounce ATR diamond crystal at 64 scans with a resolution of 4 cm^{-1} from 400 to 4000 cm^{-1}. Secondary structure of keratin was evaluated by Fourier self-deconvolution (FSD) treatment of the amide I region (1600–1700 cm^{-1}) of FTIR spectra using Omnic 8.1 software, considering a bandwidth of 26 cm^{-1} and an enhancement factor of 2.5.Characteristic secondary structure peaks in this region were compared with the reported literature.

2.7. Determination of blocked cysteine

A modified Ellman's method was used to determine the total SH group in the presence of sodium sulfite [24]. The amount of modified SH groups in NEM modified keratin was calculated according to a reported method [13] using the following equation:

$$\text{Degree of modification} = \frac{SH_k - SH_{modk}}{SH_k} \times 100 \quad (1)$$

where SH_k and SS_{modk} are the total μmol of SH of unmodified keratin and NEM-modified keratin (after dialysis and freeze drying), respectively. A molar extinction coefficient of 14,150 mM^{-1} cm^{-1} for the Ellman's chromophore molecule (5-thio-2-nitrobenzoic acid) was applied in the calculation [25].

2.8. Small amplitude oscillatory shear measurements (SAOS)

Rheological characterization of keratin gels was carried out using frequency sweeps from 1 to 10 Hz at a strain rate of 0.5%, within the linear viscoelastic region, on a Physica MCR 301 (Anton Paar, Graz, Austria). All measurements were performed at 25 °C, with a probe gap of 1 mm. To have an insight of molecular interactions, gels were soaked in different destabilizing solutions (SDS 3% w/v, urea 5 M, fresh DTT 100 mM pH 9, and urea 5 M + 100 mM fresh DTT pH 9) for 24 h at room temperature (22 °C) prior to measurement.

2.9. Zeta potential measurement

Zeta potential of keratin solutions at different pHs was measured in triplicate in a Malvern Zetasizer Nano ZSP Instrument (Malvern Instruments Ltd. UK) using 3 runs per sample.

2.10. Statistics

Experiments were performed in triplicate unless otherwise specified. Significant differences of thiol content, storage modulus and soluble protein were determined by one-way analysis of variance followed by Tukey multiple comparison test at a level of confidence of 95%. Statistics were performed using Minitab 17 software.

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