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Preparation of keratin and chemically modified keratin hydrogels and their evaluation as cell substrate with drug releasing ability

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Keratin was extracted as a reduced form from wool, which was then subjected to acetamidation, carboxymethylation or aminoethylation at abundant free cysteine residues to give acetamidated keratin (AAK), carboxymethylated keratin (CMK) and aminoethylated keratin (AEK). Hydrogels were prepared from intact and three chemically modified keratins simply by concentrating their aqueous solution and subsequent cooling. The lowest concentration to form a hydrogel without fluidity was 110 mg/ml for AAK, 120 mg/ml for AEK, 130 mg/ml for keratin and 180 mg/ml for CMK. Comparing with a hydrogel just prepared (swelling ratio: 600–700), each hydrogel slightly shrank in an acidic solution. While AAK hydrogel little swelled in neutral and basic solutions, other hydrogels became swollen and CMK hydrogel reached to dissolution. Hydrogels of keratin, AAK and AEK were found to support cell proliferation, although cell elongation on AAK and AEK hydrogel was a little suppressed. On the other hand, CMK hydrogel did not seem to be suitable for a cell substrate because of its high swelling in culture medium. Evaluation of the hydrogels as a drug carrier showed that keratin and AAK hydrogels were good sustained drug release carriers, which showed the drug release for more than three days, while the release from AEK and CMK hydrogels completed within one day. Thus, keratin and chemically modified keratin hydrogels, especially keratin and AAK hydrogels, were promising biomaterials as a cell substrate and a sustained drug release carrier.

[**Key words:** Keratin; Hydrogel; Chemical modification; Cell substrate; Sustained drug release]

The outer coverings of the vertebrate such as hair, wool, nail and skin consist of keratins, which are fibrous structural proteins belonging to the intracellular intermediate filament superfamily of about thirty molecular species (1–7). Keratins can be divided into epithelial-type keratins (soft keratins) and hair-type keratins (hard keratins). Epithelial-type and hair-type keratins are also called α-keratins to distinguish them from bird feather β-keratins, which have completely different structure and are phylogenetically distinct. Hair-type keratins are characteristically abundant in cysteine residues, which reach to 7–20% of total amino acid residues. The flexible but durable property of hair and wool is brought about by forming inter- and intra-molecular disulfide crosslinking between cysteine residues of keratins.

Previously, we reported the preparation of unmodified reduced keratins from wool simply by treating wool in an aqueous solution containing urea, 2-mercaptoethanol and SDS at 50–60°C and purifying the solubilized reduced keratins by filtration and dialysis (8). Using the reduced keratin solution, we prepared films (8,9) and porous sponges (10,11) and their physicochemical and biological properties were investigated. Keratins were found to be easily air-oxidized to give disulfide linkages on the drying process of the product, thereby, the products from keratins became insoluble in water without any treatment such as chemical and UV crosslinking. Furthermore, keratins have little risk of contamination with pathogenic agents such as virus, prion and so forth, since wool as well as hair are the tissue without the vascular systems. Free cysteine residues also gave reactive sites for modification with chemical functional groups (12–14) and bioactive substances (15). These are the advantage of keratins over other protein materials such as collagen, fibroin and so forth. The animal cells adhered, spread and proliferated well on keratin products, which might partly attributed to the presence of cell adhesion sequences such as RGD and LDV in some molecular species of keratins (10). Hydrogels from human hair keratins were intensively studied by Van Dyke and co-authors (16–20). They once pulverized keratins extracted from human hair by reduction or oxidation methods and prepared hydrogels by adding water to reduced or oxidized keratin powder. Thus prepared human hair keratin hydrogels were reported to accelerate nerve regeneration in mouse peripheral nerve injury model (16,17) and to be effective as a hemostatic agent in a rabbit lethal liver injury model (18). Here, we modified keratins at abundant cysteine sulfhydryl groups with three kinds of chemical residues with different electrical charges and prepared the intact and chemically modified wool keratin hydrogels simply by concentrating their aqueous solutions and subsequent cooling. So far, there have been no reports describing the hydrogels of keratins having modification. The physicochemical properties of thus obtained hydrogels were investigated. The ability of the resultant hydrogels as a cell culture substrate and a drug carrier, both of which are required for the cell scaffolds used in tissue engineering, were also evaluated.

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MATERIAL AND METHODS

Extraction of keratins from wool Keratins were prepared from wool according to the method reported previously (8). Briefly, keratins were extracted from 27 g of wool by incubating in 300 ml of aqueous solution containing 8 M urea (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.17 M SDS (Wako Pure Chemical Industries), and 1.66 M 2-mercaptoethanol (Wako Pure Chemical Industries) at 60°C for 24 h. After the reaction mixture was filtered through stainless mesh, the solution containing wool keratins was thoroughly dialyzed against 3000 ml of distilled water for 3 days by changing outer solution 4 times a day. Thus obtained aqueous keratin solution (15 mg/ml × 900 ml) was stored at 4°C until use.

Chemical modification of keratins Chemical modification of cysteine residues in keratins was carried out using aqueous keratin solution according to the method reported previously (21). Keratin solution (15 mg/ml × 900 ml) was reacted with 24 mmol iodoacetic acid, 8 mmol iodoacetamide or 10 mmol 2-bromoethylamine hydrobromide (Wako Pure Chemical Industries) with stirring. Reaction time was 2 h for iodoacetic acid as well as iodoacetamide and 16 h for 2-bromoethylamine. During the reaction, the pH of the solution was kept at 8.5 by adding Trizma base (Sigma–Aldrich Co., St. Louis, MO, USA) at an appropriate time. Then, the reaction mixture was thoroughly dialyzed against 3000 ml of distilled water for 1 day by changing outer solution 4 times to remove chemical reagents. The extent of chemical modification was estimated by determining remained SH residues according to the established protocol (22).

Hydrogel preparation of intact and chemically modified keratins Intact and chemically modified keratin solutions were concentrated to about 70 mg/ml of protein using an ultrafiltration unit (UHP-62K, Advantec, Tokyo, Japan) with a filter (P0200 062E, Advantec) under 2 MPa backpressure of nitrogen. Protein concentration was determined by Lowry method with a colorimetric microdetermination kit (Sigma–Aldrich). Each solution was further concentrated to 120–180 mg/ml in water bath at 60°C. Then, the solution was cast into appropriate molds and kept at 4°C for 1 day. To determine the concentration to form a hydrogel, 1 ml of keratin solution was poured into a glass cylindrical sample tube (10 mm diameter, 35 mm height). A cylindrical Teflon mold (85 mm diameter, 10 mm height) having 6 holes of 20 mm diameter placed in a 10 cm petri dish was used to prepare a hydrogel for elastic modulus measurement to give a hydrogel of 10 mm height and 20 mm diameter. For other experiments, hydrogels were prepared by pouring 48 ml of keratin solution to 10 cm plastic petri dish. After cooling at 4°C for 1 day, a hydrogel of cylindrical shape was bowled with a cork bowler to give a cylindrical shape hydrogel (6 mm height and 10 mm diameter).

Solubility test of hydrogels The solubility of hydrogels was tested by soaking a hydrogel in 50 ml of 8 M urea, 0.26 M SDS or 1.66 M 2-ME at room temperature for 1 day.

Elastic modulus measurement Cylindrical shape of hydrogel (10 mm height, 20 mm diameter) prepared as described above was subjected to the compression test using Texo Graph (UBM, Japan). A plunge of 0.5 cm^2 surface area was used at 0.05 mm/s of descending rate.

Swelling of hydrogels Each hydrogel was immersed in acetate buffer (pH 3.6), phosphate buffer (pH 7.4) and carbonate-bicarbonate buffer (pH 10.6) at 37°C until they reached a constant weight. Typically, complete equilibrium was obtained for 2 days. The weight of swollen hydrogel was measured after the moisture on the surface was removed with a filter paper. The swelling ratios (SRs) of hydrogels were calculated using $SR = (Ww - Wd)/Wd$, where Ws and Wd are the weight of the swollen and dried hydrogels, respectively.

Cell cultivation on hydrogels Intact and chemically modified keratin hydrogels were prepared on a 24- or 96-well polystyrene plate by casting 200 or 10 μl of keratin solution (150 mg/ml) into the wells of the plate and subsequent cooling at 4°C for 24 h. Each hydrogel on a dish was sterilized with 70% ethanol for 2 h at room temperature and washed with PBS for 2 h at 37°C after brief rinsing. After a hydrogel was incubated in DMEM containing 10% FBS for 3 h, the medium was replaced with fresh one and a hydrogel was further incubated for 16 h. L929 fibroblast cells were routinely cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin in T-25 plastic cell culture flasks and subcultured every 3 d. The cells at logarithmic growth phase were treated with 0.05 % trypsin-0.02% EDTA at 37°C for 5 min, washed with DMEM containing 10% FBS, collected by centrifugation at 1000 rpm for 3 min and re-suspended in DMEM containing 10% FBS. L929 fibroblast cells were seeded onto 24- or 96-well dish at 7.5×10^4 or 6.0×10^3 cells/well, respectively and cultivated at 37°C in a humidified 5% CO_2 incubator. Adhesion and proliferation of cells on 24-well dish were observed under a microscope (CKX41-31 PHP, Olympus, Tokyo, Japan). The cell number on 96-well dish was counted by a hemocytometer after detaching the cells using 0.05% trypsin-0.02% EDTA. Results of three measurements were averaged.

Drug release from keratin and chemically modified keratin hydrogels Salicylic acid, *p*-acetamidophenol and aminopyrine were chosen as model drugs. Each compound was dissolved in 5 mM Tris–HCl (pH 7.4) to produce 2 mM solution, which was then adjusted at pH 7.4 using Trizma base. Hydrogels were soaked in 10 ml of a drug solution at 4°C for 1 day and briefly rinsed with distilled water. The drug release was evaluated by soaking the drug-loaded hydrogels in 50 ml of PBS at 37°C. The amount of salicylic acid, *p*-acetamidophenol and aminopyrine released into PBS was estimated from the absorbance at 296 nm (ε: 3450) [17], 243 nm (ε: 10,300) and 260 nm (ε: 7800), respectively. UV spectra were collected using a U-2010 spectrophotometer (Hitachi High Technologies, Tokyo, Japan). To measure total amount of drug loaded on a carrier, a drug-loaded carrier was soaked in 1 M NaOH (15 ml) for 1 day to dissolve a hydrogel. After neutralization, the amount of a drug was estimated from the absorbance of each solution.

RESULTS AND DISCUSSION

Preparation and characterization of keratin and chemically modified keratin hydrogels Keratins were extracted from wool as a reduced form according to the method described previously (8). SDS PAGE analysis of thus obtained keratins showed two main bands corresponding to 52K and 62K and minor bands observed less than 14K (8). Reduced keratins were subjected to carboxymethylation, acetamidation and aminoethylation to give the residues with negative, neutral and positive charges at free cysteine residues, which reaches to about 10 mol% of amino acids in keratins, by the reaction with iodoacetic acid, iodoacetamide and bromoethylamine, respectively (Fig. 1). The amount of SH residues of keratins were evaluated before and after chemical modification by Ellman reaction (22). As shown in Fig. 2, SH residues of keratins decreased to about 5% by carboxymethylation and 15% by acetamidation and aminoethylation, indicating that most of SH residues were chemically modified. The intact, carboxymethylated, acetamidated and aminoethylated keratins were referred as K, CMK, AAK, and AEK, respectively. K, CMK, AAK, and AEK aqueous solutions were appropriately concentrated and poured into a cylindrical glass tube with a tight lid, which were then stored at 4°C overnight. At 4°C, K above 110 mg/ml, AAK above 100 mg/ml, AEK above 90 mg/ml and CMK above 170 mg/ml lost fluidity by forming hydrogels. After standing at ambient temperature for more than 4 h, K above 130 mg/ml, AAK above 110 mg/ml, AEK above 120 mg/ml and CMK above 180 mg/ml did not recover fluidity, as shown in Fig. 3. Since CMK, AAK, and AEK, which were subjected to modification on cysteine residues, formed hydrogels similarly to K, hydrogel formation might occur irrelevantly to disulfide formation. When a Teflon mold or plastic petri dish with a loose lid was used as a mold, each hydrogel was formed at a little lower concentration because of evaporation of water. When hydrogels (150 mg/ml for K, AAK, AEK and 180 mg/ml for CMK) were soaked in 8 M urea, 0.26 M SDS or 1.66 M 2-mercaptoethanol (2-ME) solutions, each hydrogel dissolved only in 8 M urea (data not shown), suggesting that K, CMK, AAK, and AEK hydrogels were possibly formed by the intermolecular interaction such as hydrogen bonds and so forth, and not by disulfide crosslinking. AAK formed a hydrogel at a little lower concentration than K, while CMK had to be concentrated to higher concentration to form hydrogel. Acetamide residues in AAK might take part in hydrogen bond formation, by which AAK hydrogel was thought to be easier to form a hydrogel. Since carboxymethylation further lowered isoelectric point (pI) of keratin which was reported to be 4.7–5.4 by Marshall (23) and 4.9–5.4 for type I keratin and 6.5–8.5 for type II keratin by Bowden et al. (24), higher concentration was needed for hydrogel formation because of intermolecular repulsion of anionically charged CMK and higher hydrophilicity. AEK formed a hydrogel at almost similar concentration to K, in spite of the introduction of aminoethyl residues. The pI of AEK was thought to be almost neutral from the calculation based on the number of cysteine residues and charged amino acids residues. The decreased intermolecular electric repulsion of AEK at neutral pH might make hydrogel formation easier. CMK and