

A BIODEGRADABLE KERATIN BASED BIOPLASTIC FILM FOR PACKAGING

Field of Invention

- 5 The present disclosure relates to bioplastic films and more particularly relates to biodegradable keratin based bioplastic films for packaging.

Background

- 10 The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

15 Plastics production has been on upward trajectory since its inception in 1950. By 2021, it was estimated that 9 billion metric ton (Mt) of plastics have entered the use phase. Single use packaging contributes to approximately 42% of plastic material usage and is the major contributor to global plastic waste. Annually, 183 Mt of solid plastics waste was estimated to enter the environment with only a small fraction of these wastes (e.g. PET-based) being fully degradable in nature, leaving the bulk as a major environmental burden. Although progress
20 has been made to develop polymers with biocleavable backbones to confer biodegradability, many of these proclaimed biodegradable plastics are still not sufficiently decomposed in real soil and aquatic ecosystems, resulting in the increase in microplastics in the environment. Considerations of sustainability and possibility to develop circular economy approaches have led to dedicated efforts to upcycle unwanted wastes from food and/or agricultural sectors to produce biopolymer-based plastic bag materials (bioplastics) which could be fully degraded in
25 nature.

Keratinous and cellulosic wastes are highly abundant and are underutilized for functional purposes. Globally, more than 10 million tons/year of keratinous wastes (e.g. feathers, hairs,
30 hoofs, horns, hair, and scales of animals) are generated each year. Hair proteins extracted from the cortex are theoretically easier to be extracted and have been proved to be promising potential biomaterials in various fields such as biomedical, pharmaceutical, and wound healing. Within the cortex, there are two main groups of proteins: (1) keratins (MW 40–60 kDa) with 50-60% in composition, and (2) matrix proteins (MW 10–25 kDa) with 20-30% in composition
35 (T. Fujii *et al.*, *Biol. Pharm. Bull.* **2004**, 27, 89-93). Keratin protein extracted from the hair cortex possess unique secondary structures and abundant thiol groups which facilitates intermolecular disulphide crosslinking, resulting in self-assembled films with great intrinsic

mechanical properties. However, although hair protein-based biomaterials have been reported extensively, they are too fragile for practical use.

Therefore, there exists a need for new biodegradable keratin based bioplastic films.

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Summary of Invention

Aspects and embodiments of the invention are provided in the following numbered clauses.

- 10 1. A composite film, comprising:
proteins comprising keratin having a molecular weight of from 10 to 60 kDa; and
a cellulose component selected from one or both of cellulose nanocrystals having a
diameter of from 175 to 300 nm and cellulose nanofibrils having a diameter of from 50 to 100
nm,
15 wherein the keratin and cellulose nanofibrils are distributed homogeneously within the film.
2. The composite film according to Clause 1, wherein the proteins comprising keratin are
present in an amount of from 5 to 99 wt% of the total weight of the composite film, and the
cellulose nanofibrils are present in an amount of from 1 to 95 wt% of the total weight of the
20 composite film.
3. The composite film according to Clause 2, wherein when the cellulose component is
only cellulose nanofibrils, the proteins comprising keratin are present in an amount of from 15
to 95 wt% (e.g. from 25 to 90 wt%, such as from 50 to 80 wt%, such as about 75 wt%) of the
25 total weight of the composite film, and the cellulose nanofibrils are present in an amount of
from 5 to 85 wt% (e.g. from 10 to 75 wt%, such as from 20 to 50 wt%, such as about 25 wt%)
of the total weight of the composite film.
4. The composite film according to any one of the preceding clauses, wherein when the
30 cellulose component is only cellulose nanofibrils, one or more of the following apply:
(a) the composite film has an ultimate tensile strength of from 30 to 40 MPa; and
(b) the composite film has a Young's modulus of from 600 to 900 MPa.
5. The composite film according to Clause 2, wherein when the cellulose component is
35 only cellulose nanocrystals, the proteins comprising keratin are present in an amount of from
15 to 95 wt% (e.g. from 25 to 90 wt%, such as from 50 to 80 wt%, such as about 75 wt%) of
the total weight of the composite film, and the cellulose nanofibrils are present in an amount

of from 5 to 85 wt% (e.g. from 10 to 75 wt%, such as from 25 to 50 wt%) of the total weight of the composite film.

6. The composite film according to any one of the preceding clauses, wherein when the cellulose component is only cellulose nanocrystals, one or more of the following apply:
- 5 (a) the composite film has an ultimate tensile strength of from 40 to 80 MPa, such as about 65.77 MPa;
- (b) the composite film has a Young's modulus of from 800 to 1,200 MPa, such as about 1,059 MPa;
- 10 (c) a water contact angle of from 60 to 80°, such as about 72.61°; and
- (d) a root mean square surface roughness of from about 10 nm to about 50 nm, such as from about 15 nm to about 30 nm (e.g. about 22 nm).
7. The composite film according to any one of the preceding clauses, wherein the proteins comprising keratin comprise keratin having a molecular weight of from 40 to 60 kDa
- 15 and matrix proteins having a molecular weight of from 10 to 25 kDa.
8. A delivery vehicle for agricultural products, comprising an outer packaging formed by the composite film as described in any one of Clauses 1 to 7 and a material for delivery.
- 20 9. The delivery vehicle according to Clause 8, wherein the material for delivery is selected from one or more of a compost, a fertiliser, and an agrifeed.
10. A degradable bag formed of the composite film as described in any one of Clauses 1 to 7.
- 25 11. A method of making a composite film according to any one of Clauses 1 to 7, the method comprising the steps of:
- (i) providing a homogeneous mixture comprising:
- 30 proteins comprising keratin having a molecular weight of from 10 to 60 kDa;
- cellulose nanofibrils having a diameter of from 50 to 100 nm; and
- water; and
- (ii) allowing the water to evaporate to provide the composite film.

35 **Drawings**

Fig. 1 depicts the Keratin/Cellulose bioplastic materials with compositions as stated in Table 1.

Fig. 2 depicts (a) physical images of keratin/Cellulose Nanocrystal (CNC) solutions with increasing concentrations of CNC from 0, 2, 4, 6, 8 and 10 wt%. (b) The graphical plot of zeta potential measurements of keratin/CNC at different concentrations of CNC depicting the colloidal stability.

Fig. 3 depicts a graphical plot of contact angle of keratin/CNC films to evaluate wettability. The inset photograph of the sessile drop of 3 μ L water has been included along with each sample.

Fig. 4 depicts stress-strain curves from tensile tests of keratin/CNC films.

Fig. 5 depicts atomic force microscopy (AFM) images of keratin films with CNC and calculated root mean square roughness (Rq) value.

Fig. 6 depicts Fourier Transform Infrared Spectroscopy (FTIR) graph plot of keratin/CNC samples.

Fig. 7 depicts photographs of keratin and keratin/CNC samples.

Fig. 8 depicts the tunability of a keratin based bioplastic film. Mechanical strength improvement and film thickness could be achieved by tuning keratin concentration.

Fig. 9 depicts the field emission scanning electron microscopy (FESEM) images of a keratin film: (a) surface morphology; and (b-d) cross-sectional morphology.

Fig. 10 depicts the water contact angle images which show the wettability of the surfaces of a keratin film and a total hair proteins (THP) film.

Fig. 11 depicts the swelling test results of a keratin film: volume change in percentage (left); and area change in percentage in the comparison of top surface area and cross-section area (right). (* is referring to significant different at $p < 0.05$, One-way ANOVA).

Fig. 12 depicts the circular dichroism (CD) spectra of a keratin solution.

Fig. 13 depicts the ratio percentage of secondary structures after FTIR deconvolution for (a) THP film, and (b) keratin film, and (c) composition percentage of protein secondary structure for both samples. (* $p < 0.05$, t-test).

- 5 **Fig. 14** depicts that (a) prolonged incubation in aqueous environment (up to 3 weeks) resulted in no perceivable change in the film integrity, highlighting the film's hydrostability. (b) Rapid degradation of the bioplastic film was observed in garden soil environment after 6 days.

Description

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It has been surprisingly found that the present disclosure provides fully biodegradable composite films made from upcycling of nature derived biowastes. Furthermore, these films have controllable physical and mechanical properties.

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Therefore, in a first aspect of the invention, there is provided a composite film, comprising:
proteins comprising keratin having a molecular weight of from 10 to 60 kDa; and
a cellulose component selected from one or both of cellulose nanocrystals having a diameter of from 175 to 300 nm and cellulose nanofibrils having a diameter of from 50 to 100 nm,

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wherein the keratin and cellulose nanofibrils are distributed homogeneously within the film.

In embodiments herein, the word "comprising" may be interpreted as requiring the features mentioned, but not limiting the presence of other features. Alternatively, the word "comprising" may also relate to the situation where only the components/features listed are intended to be present (e.g. the word "comprising" may be replaced by the phrases "consists of" or "consists essentially of"). It is explicitly contemplated that both the broader and narrower interpretations can be applied to all aspects and embodiments of the present invention. In other words, the word "comprising" and synonyms thereof may be replaced by the phrase "consisting of" or the phrase "consists essentially of" or synonyms thereof and *vice versa*.

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The phrase, "consists essentially of" and its pseudonyms may be interpreted herein to refer to a material where minor impurities may be present. For example, the material may be greater than or equal to 90% pure, such as greater than 95% pure, such as greater than 97% pure, such as greater than 99% pure, such as greater than 99.9% pure, such as greater than 99.99% pure, such as greater than 99.999% pure, such as 100% pure.

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As used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a composition” includes mixtures of two or more such compositions.

- 5 When described herein, the term “film” is intended to refer to a thin layer of material that has a thickness of from 10 to 1,000 μm , such as from 50 to 200 μm , such as 100 μm . For the avoidance of doubt, when multiple numerical ranges are provided in relation to a feature of the current invention, it is explicitly contemplated that the disclosed numerical values can be combined in any way possible to generate further numerical ranges in relation to that feature.
- 10 As an example, the values above are explicitly intended to disclose the following ranges:
10 to 50 μm , 10 to 100 μm , 10 to 200 μm and 10 to 1,000 μm ;
50 to 100 μm , 50 to 200 μm and 50 to 1,000 μm ;
100 to 200 μm and 100 to 1,000 μm ; and
200 to 1,000 μm .
- 15 For example, in some embodiments of the invention that may be disclosed herein, the film may have a thickness of $30.39 \pm 4.5 \mu\text{m}$. In other embodiments of the invention that may be disclosed herein, the film may have a thickness of $56.63 \pm 5.9 \mu\text{m}$.
- 20 The term “keratin” as used herein refers to the protein keratin, which is a fibrous protein found in hair, nails, and skin. In embodiments, any known keratin may be used herein. For example, keratins of type I and II may be used herein in any combination. In certain embodiments, the keratin may be alpha-keratin.
- 25 The term “cellulose component” as used herein to refer to cellulose nanocrystals and/or cellulose nanofibrils.

- In some embodiments of the invention that may be disclosed herein, the proteins comprising keratin have a molecular weight of from 10 to 60 kDa. The cellulose component is selected
- 30 from one or both of cellulose nanocrystals having a diameter of from 175 to 300 nm and cellulose nanofibrils having a diameter of from 50 to 100 nm. For example, the cellulose component may be cellulose nanocrystals having a diameter of from 175 to 300 nm, or the cellulose component may be cellulose nanofibrils having a diameter of from 50 to 100 nm. Alternatively, the cellulose component may be both cellulose nanocrystals having a diameter
- 35 of from 175 to 300 nm and cellulose nanofibrils having a diameter of from 50 to 100 nm. It will be appreciated that the keratin and cellulose nanofibrils are distributed homogeneously within the film.

The proteins comprising keratin may be obtained from any suitable source. In embodiments of the invention that may be mentioned herein, the proteins comprising keratin may be extracted from a mammalian source. For example, extracted from mammalian hair, nails or skin. In certain embodiments, the proteins comprising keratin may be extracted from mammalian hair, for example, human hair.

In some embodiments that may be mentioned herein, the proteins comprising keratin may be present in an amount of from 5 to 99 wt% of the total weight of the composite film, and the cellulose nanofibrils may be present in an amount of from 1 to 95 wt% of the total weight of the composite film. In further embodiments that may be mentioned herein, the proteins comprising keratin may be present in an amount of 1.5 wt% or 2 wt%.

In some embodiments that may be mentioned herein, the cellulose component may be only cellulose nanofibrils. In such embodiments, the proteins comprising keratin may be present in an amount of from 15 to 95 wt% (e.g. from 25 to 90 wt%, such as from 50 to 80 wt%, such as about 75 wt%) of the total weight of the composite film, and the cellulose nanofibrils may be present in an amount of from 5 to 85 wt% (e.g. from 10 to 75 wt%, such as from 20 to 50 wt%, such as about 25 wt%) of the total weight of the composite film. In such embodiments, one or more of the following may apply:

- (a) the composite film has an ultimate tensile strength of from 30 to 40 MPa; and
- (b) the composite film has a Young's modulus of from 600 to 900 MPa.

In some embodiments that may be mentioned herein, the cellulose component may be only cellulose nanocrystals. In such embodiments, the proteins comprising keratin may be present in an amount of from 15 to 95 wt% (e.g. from 25 to 90 wt%, such as from 50 to 80 wt%, such as about 75 wt%) of the total weight of the composite film, and the cellulose nanofibrils may be present in an amount of from 5 to 85 wt% (e.g. from 10 to 75 wt%, such as from 25 to 50 wt%) of the total weight of the composite film. For example, the proteins comprising keratin may be present in an amount of from 15 to 95 wt%, such as from 15 to 90 wt%, such as from 15 to 80 wt%, such as from 15 to 75 wt%, such as from 15 to 50 wt%, such as from 15 to 25 wt%, such as from 25 to 95 wt%, such as from 25 to 90 wt%, such as from 25 to 80 wt%, such as from 25 to 75 wt%, such as from 25 to 50 wt%, such as from 50 to 95 wt%, such as from 50 to 90 wt%, such as from 50 to 80 wt%, such as from 50 to 75 wt%, such as from 75 to 95 wt%, such as from 75 to 90 wt%, such as from 75 to 80 wt%, such as from 80 to 95 wt%, such as from 80 to 90 wt%, such as from 90 to 95 wt%, such as about 75 wt%. For example, the cellulose nanofibrils may be present in an amount of from 5 to 85 wt%, such as from 5 to 75

- wt%, such as from 5 to 50 wt%, such as from 5 to 25 wt%, such as from 5 to 10 wt%, such as from 10 to 85 wt%, such as from 10 to 75 wt%, such as from 10 to 50 wt%, such as from 10 to 25 wt%, such as from 25 to 85 wt%, such as from 25 to 75 wt%, such as from 25 to 50 wt%, such as from 50 to 85 wt%, such as from 50 to 75 wt%, such as from 75 to 85 wt%. In such
- 5 embodiments, one or more of the following may apply:
- (a) the composite film has an ultimate tensile strength of from 40 to 80 Mpa, such as about 65.77 MPa;
 - (b) the composite film has a Young's modulus of from 800 to 1,200 Mpa, such as about 1,059 MPa;
 - 10 (c) a water contact angle of from 60 to 80°, such as about 72.61°; and
 - (d) a root mean square surface roughness of from 10 nm to 50 nm, such as about 15 nm to about 30 nm (e.g. about 22 nm).

Any suitable methods may be used to determine tensile strength, Young's modulus, water

15 contact angle, and root mean square surface roughness. Specific examples of suitable methods are described in the non-limiting examples disclosed herein.

In some embodiments that may be mentioned herein, the proteins comprising keratin may comprise keratin having a molecular weight of from 40 to 60 kDa and matrix proteins having

20 a molecular weight of from 10 to 25 kDa. In certain other embodiments, the proteins comprising keratin may comprise (or consist essentially of) keratin. That is to say that the proteins comprising keratin may be free from matrix proteins, or comprise only trace amounts of matrix proteins, for example comprise less than 10 wt% matrix proteins (e.g. less than 5 wt%, less than 2 wt%, less than 1 wt% matrix proteins).

25 When described herein, the term "matrix proteins" is intended to refer to keratin associated protein (KAP).

In a second aspect of the invention, there is provided a delivery vehicle for agricultural

30 products, comprising an outer packaging formed by the composite film as described in the first aspect of the invention, and a material for delivery. In some embodiments that may be mentioned herein, the material for delivery may be selected from one or more of a compost, a fertiliser, and an agrifeed.

35 In a third aspect of the invention, there is provided a degradable bag formed of the composite film as described in the first aspect of the invention.

As will be appreciated, the degradable bag is fully biodegradable. Therefore, the application of the degradable bag can reduce environmental burden and overall handling costs in comparison to traditional plastic waste.

- 5 The composite film may be formed by any suitable means. For example, in a fourth aspect of the invention, there is provided a method of making a composite film according to the first aspect of the invention, the method comprising the steps of:
- (i) providing a homogeneous mixture comprising:
 - proteins comprising keratin having a molecular weight of from 10 to 60 kDa;
 - 10 cellulose nanofibrils having a diameter of from 50 to 100 nm; and
 - water; and
 - (ii) allowing the water to evaporate to provide the composite film.

For example, the homogeneous mixture may be casted on any suitable molds and left at room
15 temperature to allow the water to evaporate to provide the composite film.

As will be appreciated, the present disclosure has the following advantages.

1. Fully biodegradable bioplastic made from upcycling of nature derived biowastes
20 Both keratin and cellulose are nature-derived biopolymers. Utilization of both of these biopolymers in the production of bioplastics material would yield fully biodegradable bioplastics at its end-of-life. The application of the disclosed bioplastic therefore is expected to contribute to significant reduction in the environment bioburden as compared to the traditional- and biocleavable- polymeric plastic materials.
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2. Abundant and easily accessible raw materials
Keratin and cellulose could be extracted from highly abundant and easily accessible keratinous and cellulosic biowastes, respectively.
- 30 3. Contribution to circular economy
Bioplastic material produced from biowastes as its main source will contribute to the development of circular economy to upcycle unwanted wastes from food and/or agricultural sectors.
- 35 4. Transferrable and tunable physical and mechanical properties
The composite films disclosed herein possess abundant functional groups, and the properties of these bioplastic materials with tunable properties to confer durability to the bioplastics during

the usage phase could be achieved through optimization of keratin and cellulose blending ratio.

5. Ease of Fabrication

- 5 The method of fabrication described herein is simple and produces a 2D bioplastic material without the need of specialized instruments. It is a straightforward self-assembly process without complicated chemical process.

Further aspects and embodiments of the invention will now be discussed by reference to the
10 following non-limiting examples.

Examples

Materials

- 15 Tris-HCl buffer, and thiourea were purchased from Sigma. Dithiothreitol (DTT) was purchased from GoldBiotechnology. Urea was purchased from Chem-Impex. Absolute ethanol was purchased from Merck. Bleached kraft pulp (NIST RM8495) for CNF production and chloroform were purchased from Sigma Aldrich. Methanol was purchased from Fisher Scientific. Na₂S•9 H₂O (98 +%, ACS reagent) was purchased from ACROS Organics.
20 Cellulose Nanocrystals (CNCs, 10.6 % slurry) was purchased from University of Maine. Thiourea, trizma base and urea were purchased from Sigma-Aldrich Pte Ltd. The dialysis membrane (Snake Skin Dialysis Tubing; 10 KDa MWCO) was purchased from ThermoFisher Scientific. Phosphate-buffered saline (PBS) solution was purchased from Hyclone.

25 Statistical analysis

- All statistical analyses were done using the Origin software. Means comparison was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc testing, where $p < 0.05$ was considered statistically significant. All samples were run in triplicates, with three measurements taken from each sample to obtain the mean value. For swelling test, mean
30 values were calculated with at least ten measurements from each sample.

Example 1

THP extraction

- 35 Human hair was collected from a local hair salon. The hair was washed twice with detergents to remove impurities and then rinsed with absolute ethanol to facilitate its drying process at room temperature. The dried hair was then soaked in a mixture of chloroform and methanol

at a ratio 2:1 overnight in a fume hood for delipidization. After the solvents were completely evaporated, the delipidized hair was cut into lengths of 1–2 mm with scissors. THP, the mixture of keratins and KAP, was extracted by incubating 40 g of the cut hair in 1 L of 0.125 M Na₂S•9 H₂O at 40 °C for 1 h. Next, the hair residues were removed by filtration and the resultant THP solution was dialyzed against DI water for the next 4 days in a cellulose tubing with 10 kDa molecular weight cut-off (ThermoFisher Scientific SnakeSkin Dialysis Tubing) to remove the remaining Na₂S. The dialyzed THP solution was then freeze-dried to obtain THP powder and stored at -20 °C until further use.

10 *Keratin extraction*

Keratin was extracted from human hair sourced from local hair salons, by following a separation protocol described previously (T. Fujii *et al.*, *J. Biol. Macromol.* **2013**, 13, 92-106). Briefly, the delipidized hair was first incubated in an extraction solution to isolate matrix proteins (keratin associated protein, KAP), which comprises of Tris-HCl buffer (pH 9.5), 8 M urea, 200 mM DTT, and 25% vol. ethanol. Matrix protein extraction was done at 50 °C for 72 hours. The remaining 'KAP-free' hair residues were left to air-dry prior to the subsequent keratin extraction. The keratin extraction was conducted in a pH 8.5 Tris-HCl buffer containing 5 M urea, 2.6 M thiourea, and 200 mM DTT, for 24 hours at 50 °C. The extracted keratins were dialyzed against DI water for 24 hours in a cellulose tubing of 10 kDa molecular weight cut-off, with water changing interval of every 2 hours. The dialyzed keratins solution was stored at 4 °C until up to 1 week.

Example 2

Cellulose nanofibril (CNF) was incorporated to enhance mechanical property, stability and hydrophilicity of the bioplastic material. CNF was obtained from wood pulp by hydrolysis and mechanical grinding as previously described (G. Pyrgiotakis *et al.*, *Cellulose* **2018**, 25, 2303-2319). Briefly, cellulose nanofibrils were produced with an ultrafine friction grinder which possesses two ceramic nonporous grinder stones possessing adjustable clearance distance or vertical gap between the two grinders. The precut cellulose fiber sheet (dry basis; 2 x 2 cm) was soaked for 10 minutes in reverse osmosis (RO) water prior to the grinding process which was operated in batch mode at 1250 rpm. The clearance distance between the grinding stones was initially adjusted to 100 µm for the first pass, to break up any large flocs of material, and subsequently reduced to 80 µm, 50 µm, 30 µm, and finally 0 µm for the first pass of material through the grinder. The true gap was not actually zero because the presence of material between the stones forces the stones apart. The grinder stone gap setting was further adjusted to -50 µm, -100 µm, and -150 µm for the next three passes. The fibers were ground into fine

particles by shearing and friction forces. The resulting material was collected and fed back to the hopper for additional passes through the grinder. Typical CNF used in the present disclosure was produced through 10–20 passes of grinding process.

5 **Example 3**

To fabricate a keratin/CNF bioplastic material, a keratin/CNF mixture was formed by adding a CNF slurry (2 %wt. or 20 mg/ml) to a keratin solution (2 %wt. or 20 mg/ml) to give a weight ratio as indicated in Table 1. The mixture thereafter was dispersed in “one pot” with the help of stirring and mild sonication. The keratin/CNF blend (14 ml) was casted on a non-stick Teflon mold (8 cm x 12 cm x 5 mm) and left to set at room temperature, and water was removed naturally through air drying to provide a keratin/CNF film. Depending on the requirement for different applications, parameters such as ratio between keratins and cellulose, concentration, and casting volume could be further optimized.

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Table 1. Compositions of keratins/cellulose bioplastic films.

Samples	Keratins/Cellulose (wt. ratio)	
	Keratins	Cellulose
0% CNF	100	0
5% CNF	95	5
10% CNF	90	10
20% CNF	80	20

Example 4

20 Fig. 1 shows the keratin/cellulose bioplastic samples (prepared in Example 3) in different ratios as stated in Table 1. After air-drying, samples which were incorporated with 0, 5 and 10% CNF exhibited smooth surface macroscopically. Increase in the cellulose concentration resulted in the increase in the overall viscosity of the suspension, and several macroscopic bubbles being observed in the formed bioplastic.

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Example 5

Understanding the characteristic properties of the material is the fundamental phase of developing new application with mechanical properties being considered as one of the

important design parameters to be adjusted to confer durability to the bioplastics during the usage phase.

Mechanical testing

5 The samples were cut in the form of dog bone specimen using ASTM D638 – 14 standards, with 63.5 mm in total length consisting of a gauge section with 9.53 mm and 3.18 mm in length and width, respectively. The samples were placed between the pneumatic grips. Testing was done using a Mechanical Tester (MTS C42) with load of 50 N and constant speed of 10 mm/min.

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Results and discussion

The ultimate strength, modulus, and strain at break of keratin and keratin/CNF films (prepared in Example 3) in wet and dry states are summarized in Table 2. The largest drawback of films made from THP (including keratins and keratin associated proteins) is that they are highly brittle which leads to poor handling and limited accessibility. The extremely brittle behavior has made total hair protein-based platforms unavailable for tensile test unless plasticizers are added. In contrast, the tensile testing results herein show that the hair keratin-based films exhibited ultimate tensile strength (UTS) at 29.79 MPa, registering almost 16 times higher UTS as compared to wool keratin-chitosan composite films with the addition of glycerol as a plasticizer (T. Tanabe *et al.*, *Biomaterials* **2002**, 23, 817-825). The introduction of CNF was demonstrated to improve overall UTS and Young's modulus of the bioplastic blend. This suggests that the mechanical properties of the bioplastic film could be tuned by adding CNF in the blend so as to confer the desired properties.

25 **Table 2.** Mechanical properties of keratin/CNF bioplastic film obtained from tensile testing.

CNF/keratin ratio [wt%]	Ultimate tensile strength (UTS) [MPa]	Young's modulus [MPa]	Strain at break [%]	Toughness [MJ/m ³]
0/100	29.79 ± 7.87	574.09 ± 258.59	13.65 ± 4.51	2.95 ± 1.08
25/75	31.18 ± 6.74	635.87 ± 128.09	11.17 ± 3.50	2.57 ± 0.87
50/50	32.36 ± 7.69	702.34 ± 219.60	10.54 ± 2.45	2.44 ± 0.57
75/25	36.48 ± 7.28	737.63 ± 166.84	11.52 ± 3.23	2.87 ± 0.74
85/15	36.83 ± 4.65	796.28 ± 224.86	12.87 ± 2.27	3.395 ± .98

100/0	51.34 ± 12.81	496.8 ± 222.96	37.49 ± 14.53	11.66 ± 7.03
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Example 6

All CNCs used were of 175-200 nm in length and 5-6 nm in width. For thin film fabrication, it is important to evaluate the colloidal stability of the precursor dispersion as the colloidal stability can affect the surface characteristics of the film. CNCs at 0 wt%, 2 wt%, 4 wt%, 6 wt%, 8 wt% and 10 wt% were respectively mixed with keratin (20 mg/mL).

CNC solutions of different concentrations were firstly prepared at different concentrations of 0 wt %, 2 wt %, 4 wt%, 6 wt%, 8 wt% and 10 wt% in deionized water by sonication for 30 minutes. Using a magnetic stirrer at 600 rpm, the keratin solutions were mixed with each concentration of CNCs separately, wherein the volume ratio of keratin/ CNC was 3:1; a total volume of 80 mL of the mixture was made, in which 60 mL of keratin solution was mixed with 20 mL of CNC suspension.

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Breakdown for the films made with 2 wt% CNC

Concentration of keratin: 20 mg/ml (2 wt%)

Concentration of CNC: 20 mg/ml (2 wt%)

2 wt% CNC in keratin refer to a 3:1 (keratin:CNC) volume ratio of the above working solutions, i.e. 60 ml of 2 wt% keratin + 20 ml of 2 wt% CNC = 80 ml of final mixture which was cast to make one film.

The final concentration in the mixture is therefore 0.5 wt% CNC and 1.5 wt% keratin. Based on total weight of the final dried composite film, it will be 25% CNC and 75% keratin.

Breakdown for the films made with 6 wt% CNC

For 6 wt% CNC, the same calculation applies as above except that the initial concentration of CNC is now 60 mg/ml. Thus, the weight ratio in the composite film in this case will be 50% CNC and 50% keratin.

Example 7

The colloidal stability of the keratin/CNC prepared in Example 6 was measured by dynamic light scattering.

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Dynamic light scattering (DLS)

The samples prepared in Example 6 were poured into a folded capillary cell electrode (DTS1070), which was then inserted into a DLS machine. The measurement was conducted using water as the medium and the Smoluchowski model was adopted to calculate the electrophoretic mobility and in turn the zeta potential values. 5 sets of readings were taken for each sample and the statistical average with standard deviation was recorded.

Results and discussion

The zeta potential values were found to be more negative with increasing concentration of CNCs and highly stable (Fig. 2), owing to the repulsion from sulphate moieties in CNCs.

Example 8

To understand the wetting behaviour of the keratin/CNC films prepared in Example 6, contact angle measurements were carried out using 3 μ L sessile water droplet. Fourteen samples of each surface were evaluated for contact angle measurements to achieve precise standard deviation.

Contact angle measurements

The water contact angle was measured at room temperature using OCA 15 Pro Dataphysics. 3 μ L of DI water was dispensed on the sample surface at a dosing rate of 1 μ L/s.

Results and discussion

The as-prepared drop-casted keratin films showed an average water contact angle of 92.17 ± 2.7 degrees, depicting a slight hydrophobic nature attributed to the amine moieties. Keratin/2 wt% CNC showed a water contact angle of 72.61 ± 2.7 degrees (Fig. 3). The CNCs, being inherently hydrophilic, were well dispersed onto the keratin fibres, thereby increasing the wettability, or reducing the contact angle of the resultant film. However, for keratin/6 wt % CNC, a water contact angle of 97.66 ± 6.3 degrees was observed (Fig. 3), similar to the native keratin film. CNCs at 6 wt% forms aggregates and hence were not evenly dispersed within the keratin film. To understand whether any effects from the microscopic glass slide on the film surface occurred, the contact angle of the bare microscopic glass slide was recorded to be 44.74 ± 5.2 degrees (Fig. 3), which is hydrophilic in nature.

Example 9

The keratin/CNC films prepared in Example 6 were taken for mechanical testing by following the protocol in Example 5.

Results and discussion

5 From the tensile test results (Fig. 4), it was found that keratin with 2 wt% CNC had the highest UTS and Young's modulus, prominently because of the well-dispersed CNC within the keratin matrix, which facilitates hydrogen bonding between CNC and keratins, along with hydrophobic/hydrophilic interface effects. The 6 wt% CNC/keratin film was brittle and showed comparatively lower UTS value. The addition of CNC has increased the Young's modulus of
10 keratin films, proving the reinforcement effect of CNC addition, although strain at break was compromised.

Table 3. Tabulated values of the stress-strain curves of keratin and keratin/CNC films.

Sample	UTS/MPa	Strain at break/ %	Young's Modulus/ MPa
Keratin	54.02 ± 17.22	13.99 ± 4.1	574.78 ± 58.9
Keratin/2 wt% CNC	65.77 ± 21.1	7.65 ± 2.3	1059.13 ± 82.66
Keratin/6 wt% CNC	27.10 ± 8.5	3.5 ± 1	970.23 ± 141

15 **Example 10**

The keratin/CNC films prepared in Example 6 were taken for AFM imaging.

AFM imaging

20 NX 10 Atomic Force microscope (Park Systems) was used to analyse the surface morphology by imaging the samples in non-contact mode. Samples were placed on a 20 mm sample holder (stainless steel disc), which was then carefully placed inside the system. The cantilever tip was then approached near to the surface. The required area and resolution along with the line rate was chosen to record the topographical images. The images were then processed
25 using XEI software, which was used to calculate the root mean square surface roughness.

Results and discussion

Root mean square surface roughness extracted from AFM imaging of keratin/CNC samples were recorded as 19.41 ± 4.1 nm, 22.24 ± 5.5 nm, and 44.44 ± 10.8 nm for keratin, keratin/2
30 wt % CNC, and keratin/6 wt% CNC, respectively (Fig. 5). Increasing the concentration of CNC resulted in increasing surface roughness values. Keratin/2 wt% CNC recorded increasing

surface roughness compared to keratin only surfaces, but resulted in reduced contact angle, as estimated for the Wenzel state of wetting behaviour for hydrophilic surfaces (Yao, C. W. *et al.*, *Appl. Phys. Lett.* **2012**, *101*, 111605). Increased contact angle of keratin/6 wt % CNC corroborates with the high surface roughness value, with maximum exposure of keratin hydrophobic moieties.

Example 11

The keratin/CNC films prepared in Example 6 were taken for FTIR spectroscopy.

FTIR spectroscopy

FTIR spectroscopy was carried out in attenuated total reflection (ATR) mode. The sample was pressed against the sample stage with a single reflection diamond element. The spectrum resolution was fixed at 4 cm^{-1} and the sample was scanned in the wavenumber range between 400 and 4000 cm^{-1} . FTIR deconvolution was done using Origin software to determine the protein secondary structure. Peaks within amide I were resolved through the second derivative.

Results and discussion

For keratin only, the peak absorbance at 3297 cm^{-1} corresponds to the primary amine groups, 1670 cm^{-1} corresponds to Amide 1 group, 1548 cm^{-1} corresponds to Amide 2 group, and 1290 cm^{-1} corresponds to Amide 3 group, which is in accordance with the reported literature (Tan, B. Y. *et al.*, *Mater. Today Commun.* **2022**, *30*, 103049). For keratin with CNC samples, absorbance peaks at 3305 cm^{-1} , 2903 cm^{-1} , 1625 cm^{-1} , 1432 cm^{-1} , and 1160 cm^{-1} correspond to OH-stretching, CH- stretching, H-O-H bending due to water, bending of D-glucose unit and sulfate moieties because of acid hydrolysis in CNC production, respectively. No other significant vibrational bonds were observed with keratin/CNC samples (Fig. 6).

Comparative Example 1

Table 4. Basic thin film parameters.

Film	Length (cm)	Breadth (cm)	Thickness (μm)	Weight (g)	Grams per Square Meter (GSM)
Keratin	16.5	20	20.3 ± 3.4	0.57	17.27
Keratin/2 wt% CNC	15.75	10	30.39 ± 4.5	0.665	42.22

Keratin/6 wt% CNC	15.5	10	56.63 ± 5.9	1.185	76.45
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Reference Example 1

Further improvement of the bioplastics' mechanical properties could also be achieved by tuning the keratin concentration (see Fig. 8). The introduction of higher keratin concentration led to increased functional groups available for intermolecular interactions. This results in a more densely packed and interconnected network with reduced chain mobility, increased rigidity and stability of the network, and overall enhancement of the film's mechanical properties.

Reference Example 2

FESEM

Morphology of a keratin film was analyzed under a FESEM (JSM-6340 F, JEOL Co., Tokyo, Japan). The cross-sectioned sample was obtained by breaking the samples into half after liquid nitrogen freezing. Prior to imaging, the sample was sputtered with platinum at 20 mA for 40 s. For FESEM imaging, accelerating voltage and current were set at 5 kV and 12 μA, respectively.

Results and discussion

Preliminary FESEM analysis of the cross-section and surface morphologies of the keratin bioplastic is shown in Fig. 9. The keratin bioplastic showed porous and sheet-like structures with dispersed granular particles, while the surface was smooth with no visible pore observed. Some spherical grooves containing granular particles were observed in the cross-sectional images (Figs. 9c and 9d), which could be contributed to the melanosomes structure typically found in hair. Interestingly, distinctive structures were observed across the surface and cross-sectional images. This finding implies that the keratin film could have undergone large-scale molecular re-arrangement during the drying process.

Reference Example 3

The water contact angle was used to characterize the surface hydrophilicity of a keratin film.

Water contact measurement

The water contact angle was measured at room temperature using a FTA32 Contact Angle and Surface Tension Analyzer (Analytical Technologies, Singapore). 6 µl of DI water was dispensed on the sample surface at 5 µl/s.

5 Results and discussion

Another human hair protein film consisting of THP which was extracted by Na₂S (P. Hartrianti *et al.*, *Stem Cells Int.* **2015**, 2015, 752424) was evaluated as a comparison to the keratin film. The human hair protein film consisting of THP is referred to as THP film herein. The water contact angle of the keratin film was $88^\circ \pm 4^\circ$ while the water contact angle of the THP film was $67^\circ \pm 1^\circ$ (Fig. 10). This indicates that the keratin film surface was more hydrophobic. The hydrophobicity of keratin film surface might be due to the smooth surface structure, which is proposed to exhibit low water retention ability. In addition, it is also possible that the hydrophobic residues were unfolded and exposed to the film surface during the air-drying process, resulting in a relatively hydrophobic surface.

15

Reference Example 4

Swelling test

Keratin films were immersed in phosphate-buffered saline (PBS) at room temperature for 5 mins and 72 h. Length and width were measured using a ruler, and thickness was measured based on the optical microscopy images by ImageJ. Percentage of volume change, as well as top surface and cross-sectional surface area, were quantified according to the following equations:

total volume changes in percentage,

$$(V_t - V_0)/V_0 \times 100\%$$

where V_t is the swollen volume measured at wet state at time t , and V_0 is the original volume at dry state; and

top surface and cross-sectional area changes in percentage,

$$(A_t - A_0)/A_0 \times 100\%$$

where A_t is the swollen area measured at wet state at time t , and A_0 is the original area at dry state.

Results and discussion

According to Fig. 11, there is no significant difference to the total volume after immersing a keratin film in PBS for 5 mins and 72 hours. This indicates that the keratin film has reached equilibrium at 5 mins. Except for the percentage change in total volume, the area changes in the top surface and cross-sectional were quantified and compared. Interestingly, the

percentage change in the cross-sectional area was significantly higher than the top surface area. This suggests that the total volume change was mainly contributed by the cross-sectional area. In addition, it also shows that the swelling of the keratin film was directional, which is mainly affected by the cross-sectional area. This is probably due to the internal structure of keratin film as shown in Fig. 9. It proposes that the porous and hierarchical structure allowed more water retention and led to a higher swollen ratio. As a comparison, the smooth top surface area was relatively restrained from swelling. This could be due to the smooth surface with no visible pores observed under SEM (as described in Reference Example 2) as well as the low surface hydrophilicity, which were unlikely to allow high water absorption.

Reference Example 5

Circular dichroism (CD) is an excellent equipment for determining the protein secondary structure of a protein rapidly. Briefly, the optical transitions of the chromophores of the amides presented in the backbone of proteins would be captured and resulted in characteristic CD spectra corresponding to specific protein secondary structures (N.J. Greenfield, *Nat. Protoc.* **2006**, 1, 2876–2890). In this study, CD was used to determine the protein secondary structures of keratins in liquid state, which represent the state before the fabrication of the keratin film. In addition, FTIR was carried out on a keratin solution and a keratin film by following the protocol in Example 11.

CD

A dialyzed keratins solution was prepared at 2 mg/ml by following the protocol in Example 1, and a quartz cuvette with an optical path length of 0.1 mm was used. The CD spectrum measurement was performed at 25 °C. Data were obtained on an AVIV 420 Circular Dichroism spectrometer in wavelength steps of 0.5 nm, ranging from 180 to 260 nm, with an averaging time of 0.1 s over 3 scans.

Results and discussion

According to the CD spectrum in Fig. 12, there is one positive band at around 190 nm and two negative bands between 210 nm and 230 nm, which represent the characteristic bands of α -helical proteins (N.J. Greenfield, *Nat. Protoc.* **2006**, 1, 2876–2890).

In addition, the ratio of the peak intensities at 222 nm and 208 nm ($\theta_{222}/\theta_{208}$) is an indication to identify the likelihood of the helical structure being isolated or formed coiled-coil structure. According to this theoretical proposition, a $\theta_{222}/\theta_{208}$ ratio less than 0.9 represents the presence

of isolated α -helix while a ratio greater than 1 indicates coiled-coil formation. In this study, the $\theta_{222}/\theta_{208}$ ratio was approximately 1. This indicates that the coiled-coil structure was preserved after extraction. Since coiled-coil structure is an important building block for keratin filaments, maintaining this structure after extraction is desired and essential for the subsequent self-assembly process.

Deconvolution of the amine I signature peak in FTIR spectrum is a semi-quantitative method for protein secondary structure analysis. The ratio percentage of each secondary structure was obtained from the area under the corresponding deconvoluted peak (Fig. 13). For the keratin film, β -sheet was dominant, followed by random coil and β -turn. Interestingly, the keratin solution consisted of α -helix as the dominant composition based on the CD result, whereas the keratin film exhibited relatively lower amounts of α -helix. In other words, it is highly possible that most of the α -helices could have transformed into β -sheet, β -turn, and random coil during the film formation process. This is supported by the presence of 3_{10} helices which were proposed to be the intermediates as a result of helix-coil transition (G. L. Millhauser, *Biochem.* **1995**, *34*, 3873-3877).

The transition from α to β phase has been suggested as a universal mechanism of filamentous α -helical proteins to exhibit advanced mechanical properties. A THP film consists of a higher β -sheet amount compared to a keratin film. The presence of the high amount of β -sheet could attribute to the high brittleness because the β -sheet was well-reported to correlate with the advanced mechanical properties of proteinaceous biomaterials (S. H. Hiew & A. Miserez, *ACS Biomater. Sci. Eng.* **2016**, *3*, 680-693). The changes in protein conformations are affected by a few factors such as mechanical stress, solvent, heat treatment, freezing temperature, and freezing duration. In this study, this might be due to the slower drying process that provided sufficient time for molecular re-arrangement. Tamada *et al.* has reported that self-aggregated fragments were partially transformed into β -sheet structures from random coil structures during silk-based sponge formation by the freeze-drying method (Y. Tamada, *Biomacromolecules* **2005**, *6*, 3100-3106). The difference in α -helical proteins between keratin solution and keratin film suggests that the transformation of protein conformation occurred during the drying process. In addition, it is suggested that the composition of protein secondary structures present in a keratin-based film changes with different weight ratio of keratins and KAP, which could potentially exhibit desired mechanical properties without compromising the flexibility through transitions of α to β phase.

Reference Example 6

The bioplastic film in Reference Example 4 was observed to exhibit significant thickness increase but limited lateral dimension change after 3 days of incubation in aqueous solution (Fig. 11). Prolonged study up to 3 weeks showed unremarkable change in film integrity (Fig. 14A), suggesting that the bioplastic film is highly hydrostable. In contrast, rapid degradation of
5 the bioplastic film was observed after only 6 days in regular garden soil environment (Fig. 14B), due to the multitude of proteolytic enzymes secreted by the soil microbiome. This highlights the key observation that the produced bioplastic is compostable in soil, which not only reduce the environmental burden but also could potentially reduce the overall handling cost as compared to traditional plastic waste (i.e. collection, disposal costs, and recycling).

Claims

1. A composite film, comprising:
proteins comprising keratin having a molecular weight of from 10 to 60 kDa; and
a cellulose component selected from one or both of cellulose nanocrystals having a diameter of from 175 to 300 nm and cellulose nanofibrils having a diameter of from 50 to 100 nm, wherein
the keratin and cellulose nanofibrils are distributed homogeneously within the film.
2. The composite film according to Claim 1, wherein the proteins comprising keratin are present in an amount of from 5 to 99 wt% of the total weight of the composite film, and the cellulose nanofibrils are present in an amount of from 1 to 95 wt% of the total weight of the composite film.
3. The composite film according to Claim 2, wherein when the cellulose component is only cellulose nanofibrils, the proteins comprising keratin are present in an amount of from 15 to 95 wt% (e.g. from 25 to 90 wt%, such as from 50 to 80 wt%, such as about 75 wt%) of the total weight of the composite film, and the cellulose nanofibrils are present in an amount of from 5 to 85 wt% (e.g. from 10 to 75 wt%, such as from 20 to 50 wt%, such as about 25 wt%) of the total weight of the composite film.
4. The composite film according to any one of the preceding claims, wherein when the cellulose component is only cellulose nanofibrils, one or more of the following apply:
 - (a) the composite film has an ultimate tensile strength of from 30 to 40 MPa; and
 - (b) the composite film has a Young's modulus of from 600 to 900 MPa.
5. The composite film according to Claim 2, wherein when the cellulose component is only cellulose nanocrystals, the proteins comprising keratin are present in an amount of from 15 to 95 wt% (e.g. from 25 to 90 wt%, such as from 50 to 80 wt%, such as about 75 wt%) of the total weight of the composite film, and the cellulose nanofibrils are present in an amount of from 5 to 85 wt% (e.g. from 10 to 75 wt%, such as from 25 to 50 wt%) of the total weight of the composite film.
6. The composite film according to any one of the preceding claims, wherein when the cellulose component is only cellulose nanocrystals, one or more of the following apply:
 - (a) the composite film has an ultimate tensile strength of from 40 to 80 MPa, such as about 65.77 MPa;

- (b) the composite film has a Young's modulus of from 800 to 1,200 MPa, such as about 1,059 MPa;
- (c) a water contact angle of from 60 to 80°, such as about 72.61°; and
- (d) a root mean square surface roughness of from about 10 nm to about 50 nm, such as from about 15 nm to about 30 nm (e.g. about 22 nm).

7. The composite film according to any one of the preceding claims, wherein the proteins comprising keratin comprise keratin having a molecular weight of from 40 to 60 kDa and matrix proteins having a molecular weight of from 10 to 25 kDa.

8. A delivery vehicle for agricultural products, comprising an outer packaging formed by the composite film as described in any one of Claims 1 to 7 and a material for delivery.

9. The delivery vehicle according to Claim 8, wherein the material for delivery is selected from one or more of a compost, a fertiliser, and an agrifeed.

10. A degradable bag formed of the composite film as described in any one of Claims 1 to 7.

11. A method of making a composite film according to any one of Claims 1 to 7, the method comprising the steps of:

- (i) providing a homogeneous mixture comprising:
 - proteins comprising keratin having a molecular weight of from 10 to 60 kDa;
 - cellulose nanofibrils having a diameter of from 50 to 100 nm; and
 - water; and
- (ii) allowing the water to evaporate to provide the composite film.