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Cryogelation of Human Hair Keratins

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Keywords: Keratin intermediate filaments, freeze-thaw, cryogels, 3D scaffolds, tissue engineering

Human hair keratins (HHK) are reportedly known for their biocompatibility and potential to regulate cell response, possibly due to the presence of the LDV (leucine-aspartic-valine) cell adhesion and signaling motifs. Together with the abundance of cysteine residues in HHK, three-dimensional HHK scaffolds are fabricated through cryogelation based on spontaneous disulfide crosslinks and non-covalent interactions. Herein, the research is directed at understanding the molecular mechanism of HHK self-assembly during cryogelation and studying the influence of cryogelation parameters on the properties of the resultant scaffolds. With successive freeze-thaw cycles, the storage modulus (G') of HHK cryogels substantially improves from 116.4 Pa at freeze-thaw cycle 3 (FT3) to 1908.7 Pa at freeze-thaw cycle 10 (FT10). Meanwhile, it was found that complete thiol-capping of HHK samples significantly inhibited cryogel formation as compared to partially or uncapped HHK samples, suggesting the dominant role of disulfide stabilization in cryogelation. Finally, uniaxial compression tests on HHK sponges demonstrate

that FT cycling, from 0 to 10, is able to improve the compression modulus of sponges by approximately twelve-folds. These findings prove that macroscale properties of HHK cryogels can be conveniently modulated by physical parameters of cryogelation and that disulfide bonding is the main stabilizing force in HHK cryogels.

Human hair keratins (HHK) are fibrous structural proteins belonging to the intermediate filament family and are known to contain the LDV (leucine-aspartic-valine) cell adhesion motif as well as, possibly, residual regulatory molecules such as transforming growth factor- β (TGF- β) and nerve growth factor (NGF).^[1, 2] Additionally, keratins have relatively high content of cysteine residues which can partake in extensive inter- and intra-molecular disulfide bonding, allowing them to self-assemble into macroscale scaffolds such as films as well as porous hydrogels and sponges.^[1, 3, 4] At the same time, these disulfide bonds could also be targeted by alkylation to control scaffold degradation rate.^[5] Evidently, natural biomaterials possess better biocompatibility and biodegradability attributed to the intrinsic bioactive characteristics such as peptide motifs and their response to *in vivo* enzymatic reactions.^[6] Nonetheless, protein-based materials of animal origin introduce the risk of xenogeneic infections and adverse immune response.^[7] Moreover, large scale production of proteinaceous scaffolds poses an economic conundrum due to limited natural protein sources and the high cost of producing recombinant proteins as a substitute. To address these concerns, a promising solution is to exploit human hair waste as an abundant and renewable source of keratin proteins to develop an autologous biological scaffold. In fact, several studies have manifested the versatility of keratins in biomedical applications including drug and cell delivery systems or as tissue regeneration templates.^[8-11]

Amongst the reported keratin-based materials, three-dimensional (3D) scaffolds – i.e. hydrogels and sponges, have received burgeoning interest due to their potential to emulate the

extracellular matrix or native tissue milieu, which cannot be epitomized by two-dimensional substrates. The porous network facilitates nutrient exchange and removal of cellular debris, thereby facilitating cell migration, interaction, attachment and proliferation.^[12] Specific to dermal wound healing applications, interconnected porosity enables moisture retention, absorption of wound exudates and gaseous exchange while the 3D structure provides mechanical integrity for dermis restoration.^[7, 13] For instance, Wang *et al.* reported that HHK hydrogels encapsulated with L929 fibroblasts could perform comparably well, relative to collagen hydrogels, in terms of supporting cell proliferation and were better at resisting graft contraction.^[14] More recently, HHK hydrogels developed by Chen *et al.* demonstrated accelerated healing and improved the survivability of rats suffering from radiation-induced injury.^[15]

Nevertheless, the weak mechanical properties of pure HHK scaffolds limit their versatility in tissue regeneration. As such, proposed strategies often leverage on the use of chemical crosslinking or the introduction of a secondary constitute material such as polyvinyl alcohol (PVA), konjac glucomannan or alginate to design a keratin composite scaffold.^[16-18] For example, Hartrianti *et al.* fabricated a keratin-alginate sponge, crosslinked by amide bonds, which could achieve a maximum compression modulus of 191.5 kPa while incorporating the bioactivity of keratins.^[17] However, the use of chemical crosslinking agents may reduce the biocompatibility of the eventual scaffold.^[19] Therefore, cryogelation may be adopted as a facile and clean scaffold fabrication technique. Cryogelation involves three main steps, namely, freezing at a suitable sub-zero temperature, holding period in the frozen state and finally thawing at an optimal temperature. During freezing, the system enters a heterogeneous phase consisting of solvent crystals and non-frozen liquid microphase (NFLMP) in which solute particles (protein molecules) are highly concentrated but remain mobile (**Figure 1a**).^[20, 21] The NFLMP is a conducive domain for protein molecules to engage in intermolecular interactions

thereby contributing to the structure of the eventual scaffold. Concurrently, immobilized solvent crystals, behaving as porogens, grow into contact with one another, resulting in the formation of interconnected pores. Hence, the combination of cryogelation and the abundance of cysteine residues in keratins is a synergistic solution to construct 3D scaffolds based on natural crosslinks. In a recent study by Cui *et al.*, HHK sponges fabricated in a similar fashion exhibited improvements in the compressive modulus of up to 4.5 kPa following nine freeze-thaw cycles.^[22] However, as with previous studies, the scaffolds were synthesized from hair proteins *in toto*, i.e. a mixture of HHK and keratin-associated proteins (KAP), which are the hair matrix proteins. Herein, our study uses purified HHKs which are enriched with the LDV motif and have enhanced physical properties compared to crude hair extracts.^[4, 23] Additionally, the present findings evince the influence of cryogelation parameters on scaffold properties and validate the contribution of disulfide stabilization in HHK cryogels.

The cryogelation process of HHK is illustrated in Figure 1b. With the onset of FT cycling, HHK aggregates begin to form and are suspended in the aqueous medium. As FT cycling continues, the aggregates tend to associate into larger clusters which eventually form a single intact piece of gel. Another noticeable feature is the distinct phase separation between the HHK gel and the surrounding aqueous medium, termed as supernatant. Figure 1c indicates the amount of HHK that remained in the supernatant after each FT cycle. It showed that HHK concentration in the original solution decreased steadily as FT cycles increased, thus, supporting the notion that HHK aggregated and phase separate out from solution. To further elucidate the increase in aggregation of HHK during cryogelation, viscosity measurements was performed on the HHK samples using an Anton Paar MCR 501 rheometer. In **Figure 2a**, a comparison between HHK samples after zero, one, three, five and ten FT cycles (FT0, FT1, FT3, FT5 and FT10 respectively) shows that the apparent viscosity of the HHK samples increased with the number of FT cycles. Due to the rigidity of FT10 samples, the gels tended to dislodge during viscosity

measurements, thus it was not feasible to obtain meaningful data for this group. For all samples, the viscosity decreased with increasing shear rates, suggesting a shear-thinning behavior that is typical of viscoelastic fluids and gels, which could be attributed to the disruption of weak interactive forces or chain entanglements between HHK at high shear rates. Assessment on the mechanical properties of aggregated samples (FT3) and cryogels (FT5 and FT10) was conducted with oscillatory rheology. The storage modulus (G') was found to be significantly higher than the loss modulus (G'') in FT5 and FT10 HHK (Figure 2b), thereby suggesting that these samples behave more like a viscoelastic gel rather than a viscous fluid.^[24] Moreover, it was observed that increasing the number of FT cycles improved G' , with the highest recorded value of 1908.7 ± 300.2 Pa in FT10 samples. Another important feature is the crossover of G' and G'' that represents a breakdown of the gel structure and a transition from an elastic to a fluid state at higher strain rates.^[25] From Figure 2b, the crossover strain value appears to be higher as FT cycle increases. These findings indicate that FT cycling causes the cryogels to become stiffer with improved structural stability.

Freezing and thawing temperatures are crucial parameters of cryogelation which could affect ice crystal formation, cryo-concentration and molecular mobility *etc.*^[26, 27] Here, three different freezing temperatures: -195 °C (liquid nitrogen, LN_2), -80 °C, -20 °C, and thawing temperatures: 4 °C (fridge temperature), 23 °C (room temperature), 50 °C, were evaluated to investigate their efficiency in inducing cryogelation of HHK (summarized in Table S1). For meaningful comparison, the reference freezing temperature was set at -20 °C, on pilot trials, while varying the thawing temperature. Like-wise, the reference thawing temperature was determined at 23 °C while varying the freezing temperature. Based on viscosity measurements of FT5 samples represented in Figure 2c, the -20 °C/ 4 °C sample appear to be the most viscous, followed by -20 °C/ 23 °C and -80 °C/ 23 °C whereas, LN_2 / 23 °C viscosity remained comparable to that of FT0. The measurement of -20 °C/ 50 °C samples was omitted as HHK particles settled

down to form a film during the thawing process. Photographs of FT5 samples (Figure 2c inset) reveal that -20°C/4°C sample had gelled, however, in -20°C/23°C, -80°C/23°C and LN₂/23°C samples, only aggregates of varied sizes were formed. In agreement, the amplitude sweep measurements of FT5 samples (Figure 2d) demonstrated that the G' of -20°C/4°C dominated other sample groups. The next highest G' values belongs to -20°C/23°C samples followed by -80°C/23°C samples. Overall, it can be propounded that -20°C/4°C condition achieved the highest cryogelation efficiency while LN₂ treatment causes water to undergo glass transition (-137 °C) resulting in vitrification instead of crystallization thus impairing the growth of ice crystals and denying NFLMP formation.^[26] Also, high thawing temperature could have introduced thermal shocking and increased the molecular mobility of HHK molecules, thereby disrupting the intermolecular interactions between HHK.^[28]

Disulfide bonds are postulated to be the main interaction mechanism between HHK molecules, leading to cryogelation. To probe this hypothesis, inhibition of disulfide bonding between HHKs was performed by inactivating thiol groups using N-ethylmaleimide (NEM), as illustrated in **Figure 3a**. Figure S4 provides a visual summary of HHK cryogelation with different NEM/free thiol molar ratios – 0, 0.22, 0.44, 0.66, 0.88, and 1.77 (R0, R0.22, R0.44, R0.66, R0.88, R1.77 respectively). To substantiate these observations, the apparent viscosities (Figure S5) of R0 and R0.22 were found to have increased after FT1, with a similar occurrence in R0.44 and R0.66 after FT3. In comparison, the viscosities of R0.88 and R1.77 only appeared to increase at FT5 and FT10. Further rheological frequency sweep analysis on FT10 samples offered corroborative evidence that G' of R0, R0.22, R0.44 and R0.66 samples were significantly higher as compared to that of R0.88 and R1.77 (Figure 3b). These results indicate that inhibiting disulfide bonding hinders cryogelation, thus affirming that it is a major interaction mechanism to enable cryogelation of HHK. Also, it can be postulated that aggregates formed in heavily-capped samples – R0.88 and R1.77, at higher FT cycles, are

ascribed to other secondary interaction mechanisms such as hydrophobic and electrostatic interactions, and hydrogen bonding between HHK. Nevertheless, the significantly higher G' of FT10 samples at R0.44, as shown in Figure 3b similarly implies that other types of secondary molecular interactions could be significant as well during the cryogelation process. A possible explanation is that partial inhibition of disulfide bonding confers greater flexibility for HHK molecules to unfold or rearrange themselves. This in turn exposes more hydrophobic domains that can participate in non-covalent intermolecular interactions. Hence, investigations into the types and extent of other stabilizing interactions between HHK during cryogelation is warranted in future studies.

To demonstrate the efficacy of cryogelation in improving the mechanical properties of HHK sponges, uniaxial compression tests were conducted on the samples using MTS Criterion model 42 electromechanical tester. The stress-strain profile of HHK FT sponges conforms to a “J-shaped” curve as represented in Figure S6. The features of the stress-strain plot can be described by a three-regime deformation mechanism beginning with pore wall bending that is represented by the initial linear elasticity. Subsequently, the microstructure undergoes elastic buckling, rendering a plateau region, and eventually densification upon complete collapse of the structure, which is characterized by a sharp increase in stress.^[29] Variation in the number of FT cycles was studied and the data revealed that increasing the number of FT cycles significantly improves the linear compression modulus of the HHK sponges, with the highest linear compression modulus of 29.5 ± 7.7 kPa recorded for HHK FT10 samples (**Figure 4a**). Similarly, the peak compressive stress of HHK FT3, FT5 and FT10, taken at 80% strain, registered a significant increase from that of HHK FT0 and FT1 (Figure 4b). The implication of this analysis is that FT cycling enhanced the crosslinking density between HHKs in the pore walls and, consequently, the bulk compressive strength of the resulting cryogels. Cryo-concentration of HHK within the NFLMP domains in the frozen state promotes compact arrangement of the

protein molecules and association through disulfide bonds or other secondary interactions.^{[22,}
^{30]} This phenomenon is repeated through FT cycling thus translating to superior resistance
against compressive loads.^[30] Moreover, the findings demonstrate that FT cycling allows
tunable mechanical properties of the HHK sponges.

To further elucidate the correlation between macroscopic behavior and microscopic properties,
microstructure analysis of the HHK sponges was performed using the JSM-7600F Schottky
Field Emission Scanning Electron Microscope (FESEM). As seen in Figure 4c, the morphology
of HHK FT0 sample appears to have regions of sheet-like lamellae albeit with interconnected
porosity. Here, it should be noted that this sample remains fluid, hence, the HHK molecules are
susceptible to ice-templating during lyophilization at -80 °C and the formation of columnar ice
crystals along the dominant freezing direction resulted in the lamellae configuration of the
prepared sponge.^[31] By inducing FT cycling on the HHK systems, the cryogel structure is
established and reinforced by natural crosslinks. As such, the inherent pore structure would be
primarily determined by the cryogelation process rather than the interference of ice crystals
during lyophilization. This distinction in morphology is evident in HHK FT3 and FT10 samples
(Figure 4d and e, respectively) whereby the pores took on a more polygonal morphology with
undefined boundaries which render interconnectivity throughout the scaffold. Both HHK FT3
and FT10 sponges possess macro and micro pores, with the macro pores measuring
approximately 100 μm wide. Apart from the crosslinking strength, microarchitecture
differences between freeze-thawed and non freeze-thawed sponges may explain the contrast in
their mechanical compression performance. Unlike the lamellae microstructure in HHK FT0,
bridging of pore walls between sheets in HHK FT sponges could complement the load bearing
capacity. Also, under high compressive strains, the walls collapse and are compacted within the
open pores which contributes to resistance against compressive loads. This effect is reflected

by the significantly higher compressive moduli at 75% strain (Figure 4a), of HHK FT3, FT5 and FT10 sponges, relative to HHK FT0 and FT1 sponges.

In a nutshell, by harmonizing the efficacy of cryogelation, as a facile crosslinking strategy, and the ability of HHK to self-assemble, it is possible to fabricate 3D porous substrates for potential biomedical applications. Unique to this strategy, the co-dependence between processing parameters and physical properties of the cryogels and sponges suggests that scaffold properties can be conveniently modulated. Increasing the number of FT cycles enhanced the crosslinks between HHK macromolecules which resulted in stiffer cryogels and sponges. Additionally, thiol-capping of HHK divulged the significance of disulfide bonds as a dominant interaction mechanism between HHK during cryogelation. However, the formation of weak aggregates in the heavily-capped samples warrants further investigation on the presence of other stabilizing forces and their relative contribution in the self-assembly of HHK. These results offer an insight on the molecular mechanism of HHK cryogelation which could propel the development of optimized HHK substrates for further *in vitro* and *in vivo* explorations.

Experimental Section

Experimental details on materials, methods and characterization are available in Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure 1: a) Schematic illustration of the molecular mechanism of HHK cryogelation and b) cryogelation of aqueous HHK solutions over ten FT cycles. c) Reduction of protein concentrations in remaining supernatants, across zero to 10 FT cycles. Protein concentrations were normalised against that of HHK FT0 sample.

Figure 2. a) Viscosity and b) oscillatory amplitude sweep measurement of HHK samples at varied FT cycles. c) Viscosity measurement with digital images (inset) and e) oscillatory amplitude sweep measurement of HHK FT5 samples processed at different freezing and thawing temperatures.

Figure 3. a) Mechanism of thiol-capping using NEM. b) Storage modulus of HHK FT10 at varying capping ratios. * # ● Ø $p < 0.05$ compared with R0, R0.22, R0.44 and R0.66,

respectively. Comparison of means was carried out with one-way ANOVA and Tukey's post hoc test ($n=3$). c) Digital images of HHK FT10 samples at varying capping ratios.

Figure 4. a) Compression moduli measured along the initial linear region of stress-strain curves (green), * # ● $p < 0.05$ compared with FT0, FT1 and FT3 respectively; compressive tangent moduli measured at 75% strain (blue) of HHK sponges of varying FT cycles. Ø + $p < 0.05$ compared with FT0 and FT1 respectively. b) Compressive stress at 80% strain of HHK sponges of varying FT cycles. * # $p < 0.05$ compared with FT0 and FT1 respectively. Comparison of means was carried out with one-way ANOVA and Tukey's post hoc test ($n=6$). FESEM images of c) HHK FT0, d) HHK FT3 and e) HHK FT10 sponges.

Purified human hair keratin intermediate filament proteins crosslink naturally when subjected to successive freeze-thaw cycles (cryogelation) and are able to form 3D porous scaffolds. Modulation of cryogelation parameters such as the freezing and thawing temperature and number of freeze-thaw cycles enables tunable scaffold properties. Combined with the biocompatibility of keratin, these scaffolds can be potentially used in biomedical applications.

Keyword Keratin intermediate filaments, freeze-thaw, cryogels, 3D scaffolds, tissue engineering

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Supporting Information

Cryogelation of Human Hair Keratin Intermediate Filament Proteins

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Materials

Chloroform (ReagentPlus®, $\geq 99.8\%$), ethanol, thiourea ($\geq 99\%$) and N-Ethylmaleimide (NEM) were purchased from Sigma Aldrich. Methanol and SnakeSkin dialysis tubing (10K MWCO) were obtained from ThermoFisher Scientific. Urea (99.5 - 100.5%), 1,4-Dithio-DL-threitol (DTT, $\geq 99\%$) were purchased from Chem-Impex.

Experimental Section

HHK extraction and purification: Human hair, obtained from local salons in different batches, was extensively washed with detergent and delipidized in a mixture of chloroform and methanol (2:1 volume ratio). Initial removal of keratin associated proteins (KAP) was proceeded by adding delipidized hair (2.5 g) to the extraction solution (50 mL) containing Tris-HCL (2.5 mM, pH 9.5), urea (8 M), DTT (200 mM), absolute ethanol (25% v/v) in deionized water and incubated at 50 °C for 72 h.. The resulting mixture was filtered to obtain KAP-free hair as the residue. The KAP-free hair was thoroughly rinsed with DI water and left to dry at room temperature. Subsequently, HHK extraction was performed by immersing KAP-free hair (2.5 g) in a “Shindai” extraction solution (50 mL) comprising Tris-HCl (25 mM, pH 8.5), thiourea (2.6 M), urea (5 M) and DTT (200 mM) in deionized water.^[1] The mixture was incubated at 50 °C for 24 h and eventually filtered using filter paper to collect HHK extracts as the filtrate. To enable removal of extraction reagents, the filtrate was extensively dialyzed against deionized water, using SnakeSkin™ Dialysis Tubing (10K MWCO).

395

396 *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*: Samples were
397 prepared by mixing HHK extracts in NuPage® LDS sample buffer and NuPage® sample
398 reducing agent. Gel electrophoresis was conducted using NuPage™ 4-12% Bis-Tris gel at 150
399 V in NuPage® MES SDS running buffer with the addition of NuPage® Antioxidant. Upon
400 completion, the gel slab was rinsed, fixed in ethanol (40% v/v) and acetic acid (10% v/v) fixing
401 solution and stained with QC Colloidal Coomassie (Bio-Rad) at room temperature.

402

403 *Western blot*: The iBlot2 system was used for dry transfer of proteins from the gel slab onto a
404 nitrocellulose membrane for 7 min. Blocking was carried out in the presence of bovine serum
405 albumin (BSA, 3% w/v) and Tween-20 (0.05% v/v) in PBS (PBST). Next, the membrane was
406 washed with PBST before incubating with Mouse monoclonal AE13 primary antibody
407 overnight at 4°C. After rinsing with PBST, the membrane was incubated with HRP-conjugated
408 Goat anti-mouse secondary antibody in PBST (1:100 000 dilution). Finally, the
409 chemiluminescence signals were generated using SuperSignal™ West Pico PLUS
410 Chemiluminescent Substrate and imaged using ChemiDoc XRS+ imaging system.

411

412 *HHK cryogel and sponge fabrication*: HHK solutions were diluted to 20 mg ml⁻¹ with deionized
413 water and added into respective gel casting templates. Concentration of HHK was determined
414 using Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific) and referenced
415 against Pierce™ pre-diluted bovine serum albumin (BSA) standards. Next, the solutions were
416 subjected to successive FT cycles to induce cryogelation. For each cycle, the HHK sample was
417 frozen at -20 °C for 14 h and thawed at 23 °C, room temperature, (or 4 °C) for 4 h. Freeze-thaw
418 cycling was performed up to one, three, five and 10 cycles (FT1, FT3, FT5, FT10 respectively).
419 HHK FT samples in 24 well-plates were frozen at -80 °C for at least one day and lyophilized to
420 form sponges.

N-ethylmaleimide (NEM) thiol capping: Prior to the addition of NEM, protein concentration and thiol concentration of HHK were measured with BCA and Ellman's assay respectively. Thiol concentration was normalized against protein concentration. NEM solution was prepared by dissolving the reagent in deionized water immediately before capping. Also, the pH of HHK was adjusted with NaOH when necessary. Finally, HHK was simultaneously diluted to 20 mg ml⁻¹ by reacting with NEM and deionized water to achieve the various NEM/free thiol ratios (0, 0.22, 0.44, 0.66, 0.88, and 1.77). To ensure effective capping, the samples were incubated for 2 h at room temperature. Thereafter, the capped samples were dialyzed against deionized water to remove excess NEM and the samples were eventually subjected to FT cycling.

Rheological measurements: Tests were conducted using the Anton Paar MCR 501 rheometer equipped with a temperature control unit. Fluid samples were measured with a cone plate (CP25-1). Approximately 70 µl of the sample was pipetted onto the rheometer stage below the spindle with a fixed measurement gap of 0.047 mm. For gel and paste-like samples, measurements were done using a parallel plate (PP10) spindle of 10 mm diameter. Depending on the sample thickness, the PP10 spindle was lowered to a gap between 0.5 – 2 mm. In this step, the normal force on the sample was maintained between 0.02 – 0.05 N. Under the controlled shear rate regime, the shear rate was set to ramp from 0.0001 – 1000 s⁻¹ in logarithmic steps. In oscillatory rheological measurements, the amplitude sweep was carried out by increasing the strain from 0.01 – 1000% in logarithmic steps at a constant frequency of 6.28 rad/s (1Hz). Based on the plot of G' and G'', a constant strain value of 0.5% was chosen for subsequent frequency sweep tests. The angular frequency was applied over the range of 0.628 rad s⁻¹ (0.1 Hz) to 75.36 rad s⁻¹ (12 Hz). This range of frequencies was selected because of its physiological relevance to human skin.^[2]

Mechanical compression: Uniaxial compressive loading was assessed using an electromechanical tester (MTS Criterion model 42). The equipment was fitted with a 50 N load cell. The dimensions of the sponges are 5 – 6 mm thick and 15 mm in diameter. For each run, the sample was placed between two unlubricated platens (50 mm in diameter) and was pre-loaded with 0.05 N. The sponges were then compressed at a loading speed of 0.01 mm s⁻¹ until 80% strain. The compression moduli of the sponges were obtained from the gradient of the initial linear segment and the tangent at 75% strain of the stress-strain plot. The peak compressive strength was determined by the highest compressive stress measured at 80% strain.

Scanning electron microscopy (SEM): Microarchitecture analysis of lyophilized HHK sponges was performed via SEM under the secondary electron imaging (SEI) mode. HHK solution were casted into acrylic rings which were then subjected to FT cycling and frozen at -80 °C at least one day prior to lyophilization. The sectioned sponges were mounted onto half-stubs, gold sputtered under vacuum at 20 mA for 40 s and eventually observed using a field-emission scanning electron microscope (FESEM, JEOL, JSM-6700F) at an accelerating voltage of 5 keV. Pore sizes were estimated from at least 200 measurements using ImageJ software.

Statistical analysis: Statistical analysis was performed using OriginPro 2016 software. One-way analysis of variance (ANOVA) and Tukey's post hoc test was conducted on n number of samples (indicated in results). Data was considered statistically significance where $p < 0.05$.

Supporting figures and data

Figure S1. Left – SDS-PAGE analysis of purified HHK and KAP extracts stained with coomassie blue; right – Western blot imaging of HHK extracts. Std refers to standard. The red box indicates type I and type II HHKs.

Table S1. Freezing and thawing temperature and respective duration.

	Temperature (°C)	Duration
Freezing	-20	14 h
	-80	14 h
	-195.79*	15 min
Thawing	4	>4 (including storage)
	23	4 h
	50	4h

*Liquid nitrogen (LN₂)

Figure S2. Progression in gelation of HHK during FT cycling at different freezing and thawing temperatures.

Figure S3. Normalized thiol concentration of HHK capped with different ratios of NEM.

Figure S4. Progression in gelation of HHK for different NEM/free thiol ratio from FT0 to FT10.

Figure S5. Viscosity measurements for different HHK capped with different NEM/free thiol ratio at a) FT1, b) FT3, c) FT5, d) FT10.

Figure S6. Example of stress-strain curve of HHK FT sponges.

References

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