Human hair keratin and its interaction with metal ions

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**HUMAN HAIR KERATIN AND ITS INTERACTION WITH
METAL IONS**

LOW CHOON TECK

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

HUMAN HAIR KERATIN AND ITS INTERACTION WITH METAL IONS

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Engineering

2020

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Abstract

Keratinous fibers from various sources had been used for the adsorption studies of metal ions such as Cu, Zn, Pb, Cr, Hg, Ag, and others. These fibers were found to chelate metal ions by introducing chemical treatment to the hair fibers which allows better penetration of the metal ions into the hair fiber, or by disrupting the disulfide bonds in hair which produces free thiol groups. However, there were no quantitative data to explain the improved binding of metal ions to keratinous fibers after chemical treatment. Furthermore, there were no articles that gave a direct correlation between the thiols in keratin to the binding of metal ions.

Although it is well known that thiols in cysteine bind well to metal ions, cysteine residues in proteins might have a different characteristic under different conditions. This could be due to the folding of proteins or interference from neighboring groups affecting the reactivity of thiols in cysteine. Therefore, the hypothesis of this project is that thiols in keratin are the main functional group responsible for the binding of metal ions to human hair keratin. The objectives of this project are to find out the functionality of thiol in extracted human hair keratin for the binding of metal ions.

Keratin were extracted from human hair fibers using reduction method through thiol disulfide exchange with another thiol compound. Quantification of protein and thiol concentration were done by Bradford and Ellman's assays, respectively. The assays were selected as they are less prone to interference and requires relatively short incubation time below 20 minutes. The extracted keratins were subjected to different pH treatments and it was found that an acidic pH gave the best physical behavior and highest amounts of functional thiol groups, at about 1 mmol/g. Subsequently, keratin in acidic pH were used for the interaction studies with copper (II) ions to find out the relationship between copper and thiol concentrations.

Results showed that the molar ratio between copper and thiol concentration has to be below 0.5 to prevent copper from oxidizing free thiol groups into disulfide which makes it unreactive. To further support the results of copper thiol binding in keratin, free thiol groups were capped with NEM. The capped keratin was subsequently subjected to different concentration of copper and it resulted in no interaction between copper and KIF. This result further validated the hypothesis that thiol is the main functional group in keratin involved in metal binding.

Following the mechanisms of copper thiol binding in keratin, metal ions commonly found in electronic wastes such as nickel and aluminum were mixed with copper and interacted with keratin. Scarce metal such as silver were also interacted with keratin. Results from the experiment showed that both nickel and aluminum did not affect the binding of copper to keratin. Thus the binding of keratin appears to be highly selective towards copper in the mixed metal ions solution. A possible reason might be Hard Soft Acid Base (HSAB) whereby soft acid would prefer to bind to soft ligand and vice versa for hard acid base. Another possible explanation could be due to the intrinsic properties of the metal ions such as the stability of its oxidation states in aqueous solution.

Lastly, keratin-associated proteins (KAPs) which have about 3 times more thiols than keratin were subjected to copper ions interaction. As expected, since KAPs have higher amounts of thiol, lower protein concentrations were required to achieve the same experimental outcomes of keratin.

In conclusion, In conclusion, thiol groups in keratin were functional and responsible for the binding of keratin to metal ions. Between monovalent, divalent and trivalent metal ions, monovalent silver ions had the highest affinity towards thiol whereas trivalent aluminum had the poorest affinity. Lastly, keratin associated protein (KAP) displayed better efficacy in binding copper ions as compared to keratin due to its higher cysteine content. However, more focus was placed on keratin due to the presence of cell adhesion motif, LDV (Leucine-Aspartic acid-Valine) and the ability to form fibrous structure, for downstream biomedical application.

Lay Summary

Waste generated from a barber shop can actually be valuable raw materials for a wide range of sectors from medical to engineering. Human hair are often treated with various products to give it different colors, styles and texture. These features are possible due to the changes occurring in the amino acids and proteins in hair. One of the major proteins found in human hair is keratin. An example of the most prominent and recognizable effects of keratin on the appearance of hair can be seen on a daily basis in people having either wavy or straight hair. These are achieved by modification to the interactions between keratins, particularly through the amino acid cysteine. Hence, it is important to understand the characteristics of the amino acids found in keratins as well as the behavior of keratins in different chemical and physical environment. In this project, studies were done to explore the possibility of using keratins as a material to help improve the environment by using it to extract metals from electronic wastes. Low traces of metals can be found in hair, however, it is difficult to determine if those metals arise during the formation of hair or due to environmental factors. A common occurrence where blond hair turns green in the swimming pool can be explained by the interaction between copper ions in the pool and hair fibers. Currently, available literature focuses on the interaction between human hair surface and metal ions. There is a lack of fundamental studies between the keratins in hair and their interaction with metal ions. Thus, in this project, correlations of interactions between metal ions and keratin were established, with a focus on the role played by cysteines, which contributes mostly to the chemical and physical properties of hair. Results showed that molar ratio between copper and keratins has to be controlled for interaction to occur. Apart from molar ratio between metals and keratins, other chemical and physical principles have to be considered as well. In conclusion, keratins displayed the ability to bind metal ions under certain conditions. The efficacy of keratins to bind metal ions were also higher than hair fiber due to the availability of more exposed thiol functional groups. Therefore, it will be worthwhile to continue more fundamental research on keratins interaction with metal ions for environmental applications.

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Abbreviations

2-ME	2-Mercaptoethanol
BSA	Bovine Serum Albumin
DTDP	4,4'-Dithiodipyridine
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HSAB	Hard and Soft Acid and Base
ICP-OES	Inductive Coupled Plasma Optical Emission Spectrometry
IMAC	Immobilized Metal Ion Affinity Chromatography
KIF	Keratin Intermediate Filament
KAP	Keratin-Associated Proteins
LDV	Leucine-Aspartic acid-Valine
NEM	N-Ethylmaleimide
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TGA	Thioglycolic acid

Chapter 1

Introduction

Human hair is often treated as waste which eventually ends up getting incinerated. However, within the hair fiber, there are proteins known as keratins and KAPs which play an important role in the formation of hair fiber. In this chapter, a brief introduction on the properties of keratin as well as the potential use of keratin as a biosorbent to attract metal ions are presented. The hypotheses and experimental scope to find out the interaction between keratin and metal ions are also discussed in this chapter.

1.1 Introduction

There had been an ongoing effort to conserve the environment, especially with industrialization leading to an increase in discharged pollutants into the environment. Some of the common pollutants are heavy metal ions such as copper, lead, nickel, cadmium, chromium, zinc etc [1, 2]. These heavy metal ions not only pollute the environment, but can also be absorbed by marine animals such as fishes which may end up being consumed by humans. High level exposure to heavy metal could lead to serious complications such as organ failures, neurological disorders, respiratory issues and more [1]. Therefore, it is important to mitigate the release of heavy metal ions into the environment. While there are many technologies such as membrane filter, chemical precipitation, ion exchange, electrochemical treatment [2], pyrolysis, and hydrometallurgy, some of these methods consume large amount of utilities due to the harsh conditions required. Other problems include the depletion of natural resources and usage of highly toxic chemicals which could cause land and marine eco-toxicity. Therefore, a potentially more sustainable and environmental friendly process, known as biometallurgy, is being explored as an emerging research area. Although biometallurgy would still require the use of chemicals to process the biomaterials, toxic by-products generated after the processes should be reduced [3]. Hence, the area of interests in this project is to study and understand the properties of thiol in extracted human hair keratin for the potential use in metal binding applications.

Wool, human hair, and feathers are keratin-containing materials that are often treated as waste. These materials are subjected to incineration or landfill with only a small proportion being recycled and used as fertilizer or biodegradable surfactants [4]. As keratin is a protein with high cysteine content, it is able to crosslink and form disulfide bonds which gives human hair its strong mechanical properties as well as its low solubility in water [5]. Due to the crosslinking, there are limited availability of free thiol groups in hair shafts to participate in chemical reactions, hence reductive or oxidative reactions are employed to break the disulfide bonds.

Interaction between proteins and metal ions are based on the electron rich groups of the

protein and the accessible sites of the metal ions. Affinities between the binding sites of the protein and metal ions are explained by the hard and soft acid and base (HSAB) principle. Briefly, in the binding of two atoms, one atom would act as a Lewis acid and the other as a Lewis base. Generally, hard Lewis acids would prefer to bind to hard Lewis bases and vice versa. Hard Lewis acids and bases are classified as having high charge to ionic radius while soft Lewis acids and bases would have low charge to ionic radius. Examples of hard Lewis acids are potassium ions (K^+), calcium ions (Ca^{2+}), magnesium ions (Mg^{2+}), iron (III) ions (Fe^{3+}), and aluminum (III) ions (Al^{3+}), while soft Lewis acids include silver (I) ions (Ag^+), and copper (I) ions (Cu^+), and borderline Lewis acids comprise of cobalt (II) ions (Co^{2+}), zinc (II) ions (Zn^{2+}), copper (II) ions (Cu^{2+}), and nickel (II) ions (Ni^{2+}) [6]. Following the HSAB concept, sulfur is considered as soft Lewis base which coordinates well and have strong affinity for borderline Lewis acids such as Cu^{2+} [6-8] as well as monovalent ions such as Ag^+ .

The focus of this report would be to find out the relationship between the high cysteine content of extracted human hair keratin to the removal of copper ions from a solution. The findings would be beneficial to further understand the behaviors of keratin in the presence of metal ions which could potentially lead to keratin being used as a biosorbent to purify water.

1.2 Hypothesis/Problem Statement

With the understanding of current literature, the hypothesis derived are that:

- 1) Free thiols from cysteines are the main functional group responsible for the binding of hair keratins to metal ions.
- 2) Extracted human hair keratins have higher metal ion binding efficacy than the human hair fiber itself.

1.3 Objective and Scope

The overall objective of this research is to generate a deeper understanding of the interaction between extracted human hair keratin with metal ions which could potentially be used as a biosorbent for metal ion chelation and environmental remediation applications.

To achieve the objective, scopes of this project would include:

- 1) Comparing the amount of free thiol group available against different pH ranging from acidic to alkaline.

This approach is based on the concept of thiol-disulfide exchange which either leads to the breaking of disulfide bonds producing free thiols or the formation of disulfide bonds from free thiols. Since the pK_a of cysteine is around 8.5 [9], by keeping keratin in a pH solution of < 8.5 the thiolate anions should be reduced, thus free thiols would not be oxidized into disulfide.

- 2) Interaction of keratin with different concentrations of copper ions

Based on sulfur metal chemistry, sulfur has high affinity for metal ions. Thus, keratin which has high cysteine content should be able to bind to copper ions. However, in some cases, copper ions oxidize free thiol to disulfide instead of forming copper-sulfur complex. Therefore, the relationship between the concentrations of thiols to concentration of copper ions must be understood to control the binding efficiency of keratin.

- 3) Capping of thiol in keratin to verify the functional group involved in metal binding

Since it was hypothesized that thiol is the main functional group involved in binding of metal ions to keratin, capping of thiol would be done to compare the results of metal binding between capped and non-capped keratin.

4) Interaction of keratin with solution of mixed metal ions

Metal ions might behave differently in the presence of other metal ions which could affect its reactivity. A few authors have suggested that certain metal ions might suppress or promote the binding of a particular metal in a mixed solution to human hair fiber [2, 10]. Therefore, metals commonly found in electronic waste would be mixed and reacted with keratin to find out their effects on metal-keratin binding.

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Chapter 2

Literature Review

In this chapter, an introduction to human hair fibers is provided where the different regions of hair are explained, particularly the cortex region which contributes to the bulk of hair mass. Next, the proteins that contribute to the formation of keratin intermediate filament are described beginning from 2 monomers, type I and type II keratin. Subsequently, the chemistry aspect of the proteins is discussed where the focus is mainly on thiol chemistry as keratin has high amount of thiol groups as compared to other structural proteins. Finally, a review on existing literatures that uses human hair fiber to interact with metal ions were presented.

2.1 Hair Fibers

The morphology of a fully-grown human hair can be classified into three main sections, namely, the cuticle, cortex, and medulla. The outer-most layer which provides chemical resistance properties to the hair is the cuticle [1], followed by the cortex which contributes to the bulk of the mass, and finally the medulla which is a loosely packed porous region in the hair fiber [2]. The cortex region of hair fibers consist of cells and intercellular binding materials which is responsible for the physical properties of hair such as elasticity [3, 4]. In the nanoscale, intermediate filaments surrounded by a matrix bundles together to form macrofibrils which assemble longitudinally in the cortical cells found in the cortex region [5].

Figure 1: The formation of human hair fiber starting with α -helix chain. Reprinted with permission from Elsevier [3].

The building blocks of hair fibers consist of a group of α -proteins that contributes two out of six classes of intermediate filament proteins known as type I: acidic keratins and type II: basic keratins. Both type I and type II keratins can be found in hard or soft keratins which are categorized based on the source that it was obtained from. Hard keratins are derived from hair, beak, horn, nails, and beaks, while soft keratins are derived from the epidermis and other soft epithelial tissues [6]. It has been found that there are eleven type I keratins of molecular weights between 44 kDa to 56 kDa and six type II keratins of molecular weights between 53 kDa to 65 kDa being expressed in hair

follicle [7, 8]. Hard α -keratin of hair was first named in 1986 where it was denoted as Ha and Hb followed by a number where 'H' referred to hair, 'a' referred to type I: acidic subtype, 'b' referred to type II: basic subtype [9]. Further improvements to the naming system were done to make it more systematic and durable in the long run. For human hair keratin, the 11 type I acidic keratins were numbered from 31 to 40 whereas the 6 type II basic keratins were numbered from 81 to 86 [10]. A summary of the genes and naming system of type I and type II human hair keratin are provided in Table 1 and Table 2.

Table 1: Total number of type I and type II gene in human keratin. [10]

Human keratin genes	Type 1 genes	Type II genes
Total genes	33	34
Functional genes	28	26
Pseudogenes	5	8
Epithelial keratin genes	17	20
Hair keratin genes	11	6

Table 2: The number used for naming each category of keratin. [10]

Category	Number range
Human type I epithelial keratins	9 - 28
Human type I hair keratins	31 - 40
Non-human type I epithelial and hair keratins	41 - 70
Human type II epithelial keratins	1 - 8 and 71 - 80
Human type II hair keratins	81 - 86
Non-human type II epithelial and hair keratins	87 - 120
Type II keratin pseudogenes	121 - 220
Type I keratin pseudogenes	221 ->

The formation of intermediate filaments begins with the arrangement of a heterodimer consisting an equimolar amount of type I and type II keratin, where one type II keratin will interact with one type I keratin to form a coiled-coil rope. The process continues with the aggregation of two coiled-coil rope in an antiparallel manner to form a four-polypeptide chain. Finally, the four polypeptide aggregates would arrange itself with

another four polypeptide aggregates to form an intermediate filament [11, 12]. An illustration of the formation of hair fiber is shown in Figure 1 and Figure 2 shows the dimer formation from type I and type II keratin.

2.2 Keratin Intermediate Filament (KIF) in Human Hair

Proteins, lipids, and melanin are components found in the human hair which could have some variation due to the ethnicity, location, age, and nature of the hair. It is well known that hair has high amounts of sulfur protein as well as a range of other proteins with function groups that are acidic, basic, and neutral [13]. The most abundances proteins found in hair are a group of structural proteins commonly known as keratin which is found in the cortex region of hair that bundles together to form the keratin intermediate filament (KIF) and covalently crossed linked with a matrix known as keratin-associated proteins (KAP) [14-16]. KAPs are a group of proteins that can be divided into three categories: 1) high molecular weight sulfur proteins (50 to 75 kDa, 20% cysteine residues) 2) ultra-high sulfur proteins (30 to 40 % cystine) and 3) low molecular weight glycine-tyrosine proteins (15 to 50 kDa) [17]. Changes to KAP molecular structure are thought to be linked to hair damages [14]. However, as KIFs are able to form fibrous structure which could result in more useful applications in various fields, more studies were done on the properties of KIFs as compared to KAPs [14].

Both KIF and KAP are sulfur-rich proteins because of high proportions of thiol (-SH) containing amino acid, cysteine. The thiol in most sulfur proteins are responsible for a range of function from forming structural disulfides to metal binding, redox reaction, nucleophilic reaction, etc. Therefore, most discussion relating to sulfur proteins and cysteine refers to the reaction between thiols and the target ligands. Nonetheless, different names are used depending on reference to the protein (sulfur protein), amino acid (cysteine) or functional group (thiol).

Figure 2: Schematic diagram of the interaction between type I and type II keratin to the formation of keratin tetramer. Reprinted with the permission from creative common attributed license [18].

The main differences between keratin and other structural proteins such as collagen and elastin are their high cystine content which impacts certain properties such as resistance to enzymatic lysis and insolubility. Based on the amino acid sequences of keratin, cystine content in hair can be as high as 22 % as compared to chicken feather which has about 8.2 %, and wool which has about 14.4 % [19, 20]. In addition, keratin has leucine-aspartic acid-valine (LDV) amino acid sequences that promotes cell adhesion which is beneficial for biomedical applications [21].

The physical and chemical properties of hair are largely related to the presence of disulfide in hair. For instance, in cosmetic applications, straightening or curling of hair starts with the reduction or breaking of disulfide bonds [2]. Keratin can also be made into other soluble derivatives by the reduction or oxidation of disulfide bond [22]. Keratin that was extracted via oxidation are called keratose whereas keratin

obtained from reduction are called kerateines [16, 23]. The properties of extracted keratin can be tuned to have a wide range of properties that could be useful for many applications. An example is the fabrication of hydrogel from keratin which could be used to support cell growth, drug delivery, tissue engineering and also implantation in humans [24]. Other applications include fiber composites such as clear plastic films [25], paper with different functions from controlling of humidity to adsorption of metals and many more [26].

2.3 Human Hair Keratin-Associated Protein (KAP)

Another important protein found mainly in the cortex and cuticle regions of hair are known as keratin-associated proteins (KAP). KAPs can be grouped into three categories: 1) high sulfur (< 30mol% cysteine content), 2) ultrahigh sulfur (> 30mol% cysteine content) and 3) high glycine/tyrosine KAP [27]. Currently, out of the 23 families of KAP found, 21 of them can be found in humans. Figure 3 depicts an overview of the KAPs found in the different region of a hair shaft. From the diagram, most of the KAPs are found in the cortex region of hair. Among the three categories of KAPs, high sulfur KAPs and high glycine-tyrosine KAPs are the most abundant.

Figure 3: Illustration of KAPs located in different region of the hair. Reprinted with permission from Elsevier [28].

In the hair cortex region, KIFs are embedded in KAPs which are also known as the matrix protein. It is well known that KAP acts as a crosslinker with KIF to form disulfide bonds and this is the main mechanism that holds the cortical region together [23]. Although many KAP had been discovered and isolated, their properties are still being investigated [29]. An investigation on the hydrating effect of KIF with different KAP content were conducted in 2012 by *Greenberg, D.A and Fudge, D.S.* It was hypothesized that KAP is less hydrophilic than KIF, thus the high amount of KAP would result in less swelling of hard α -keratin in water. From their research, they found that hard α -keratin hydration was influenced by the amount of matrix proteins and this finding could help in the design of novel polymer matrix which might be sensitive or insensitive to solvents [30].

A more recent study in 2017 by *Singh, R.S., et al.* [29] focused on characterization of one of the high glycine-tyrosine (HGT) protein, KAP 8.1 in human hair. It was believed that KAP largely contributed to the glassy state physics of human hair and there are not many studies done on individual KAPs. Therefore, computation modelling and experimental verification was successfully done for KAP 8.1 to understand its structure. Finally, the authors concluded that the model of KAP 8.1 would be a good starting point to further study the properties of KAP in various environmental conditions as well as its interaction with various small molecules [29]. Hence, it would be interesting to find out the correlation between cysteine in KAP and its interaction with metal ions as well as its behavior compared against KIF.

2.4 Thiol-Disulfide Chemistry

To understand and study hair, the basic building block of hair which is keratin must first be understood. For this to be possible, keratin must first be solubilized to enable further characterization. This insolubility had been found to be largely caused by disulfide bonds present in keratin. Therefore, breakage of disulfide bond is critical for the solubilization of keratin [31]. Ionic scission of disulfide bonds via a nucleophilic reagent occurs as a S_N2 reaction whereby a bond is broken and another new bond is form simultaneously. This mechanism is applicable to thiol-disulfide exchange, sulfite-alkylthiosulfate exchange, elemental sulfur reaction with

triphenylphosphine or cyanide ions and many other polythionate ions reactions [32]. The thermodynamics for nucleophilic displacement of sulfur is affected by the nucleophilicities of the nucleophiles and the stability of the leaving group. In addition, electron-withdrawing substituents attached to a sulfur will make the disulfide compounds more liable to cleavage. An example of a series of nucleophiles starting from the most nucleophilic compound are as shown: $\text{CH}_3\text{CH}_2^- > \text{C}_6\text{H}_5\text{S}^- > \text{CN}^- > \text{SO}_3^- > \text{OH}^- > \text{N}_3^- > \text{SCN}^-$, I^- [33].

To extract keratin from human hair fiber, it is essential that disulfide bonds are cleaved. A general thiol-disulfide exchange reaction is shown in equations (1) and (2) where X represents any substituent group. The reaction starts with a nucleophilic displacement by a thiolate anion ($\text{X}'\text{S}^-$) forming a mixed disulfide ($\text{XS}-\text{SX}'$). This is followed by the displacement of another thiol group from the mixed disulfide by excess thiolate anions ($\text{X}'\text{S}^-$) which give rise to a symmetry disulfide ($\text{X}'\text{S}-\text{SX}'$). The net overall results for the reaction is given as equation (3) [32].

The net result for the reaction is shown in equation (3),

A similar mechanism had been proposed for the reduction of disulfide bonds in human hair, whereby initiation of reaction starts with a nucleophilic displacement of thiol on a symmetrical disulfide ($\text{K}-\text{S}-\text{S}-\text{K}$) of hair by a thiol group ($\text{R}-\text{SH}$) as shown in equation (4). Similarly, the reaction continued with the displacement of thiol on the mixed disulfide ($\text{K}-\text{S}-\text{S}-\text{R}$) by excess thiol group ($\text{R}-\text{SH}$) as shown in equation (5). To summarize the reaction, equation (6) was generated to provide the overall equation [2].

The overall equation is shown in equation (6),

Following the concept, various reduction methods were explored to extract keratin from various sources. The common reagents used are a reductant which is used to reduce disulfide bonds, and a denaturant such as urea which is used to disrupt the hydrophobic interaction between polypeptide chains. Sodium dodecyl sulfate (SDS) was also used as a surfactant to reduce protein aggregation. Temperature and pH were the other parameters that could affect the outcome of the extraction. Increased temperature could speed up reaction kinetics, but higher temperatures could also cause amino acid to be degraded. The effect of pH is similar whereby a highly alkaline pH could cause cleavage of peptide bonds and a highly acidic pH could result in unwanted by-products formation [34]. Table 3 depicts the different reagents and conditions used for the extraction of keratin.

Table 3: Examples of keratin extraction through the reduction methods.

Thiol	Experimental conditions			Reference
	Urea (M)	Buffer used	pH	
5% 2-ME	5	3 M thiourea, 25mM Tris HCl	8.5	[35]
5% DTT	5	2.4 M thiourea, 25mM Tris HCl	8.5	[35]
250 mM DTT	5	2.6 M thiourea, 25mM Tris-HCl	8.5	[14]
0.5 M TGA	Nil	NaOH, 100 mM Tris base followed by	11	[16]

		deionized water		
0.5 M TGA	Nil	NaOH	12	[36]
1.4 M 2-ME	6	1.4g SDS, 3 mM EDTA and 0.2 M KCl-NaOH or 0.2 M NaHCO ₃ or 0.2 M Tris buffer	10 9 7	[37]
5 % v/v 2-ME	7	2 % w/v SDS	Neutral	[38]

Apart from reducing the disulfide in cystine to obtain cysteine which has the free thiol groups, it is also important to prevent the free thiol groups from re-oxidizing into disulfides causing it to become insoluble. To further manipulate the properties of keratin, keratin must be soluble thus various chemical modification of cysteine are done to block the thiol groups from re-oxidizing. One of the methods used is via alkylation of –SH, using moniodoacetamide (I-AAm), moniodoacetic acid (I-AA) or monobromosuccinic acid (Br-SA) which results in the keratin compounds shown in Figure 4 [39].

In addition to the chemicals mentioned above, modification of thiol via Michael addition using maleimides are also a popular choice due to the stability of the resulting thiol-maleimide conjugate. The reaction between maleimide and thiol occurs when a thiolate anion attacks the electrophilic C=C bond of maleimide to form a thioether bond between thiol and maleimide. The reaction between I-AAm, I-AA, Br-SA and thiol is a nucleophilic substitution (S_N2) reaction where the rate of reaction is dependent on the nucleophilic concentration, in this case the thiolate anions, concentration of the reactant (I-AAm, I-AA, Br-SA), pH and proticity of the solvent. In contrast to the S_N2 reaction, Michael addition has a faster reaction kinetics and is less influenced by the pH. However, maleimide is less specified and might take part in other side reactions with lysine and histidine residues [40]. Therefore, a comparison between the different thiol capping reagents should be done before selecting the most appropriate ones. Between the few common reagents, N-ethylmaleimide (NEM) was found to be more effective than I-AAm and I-AA as less

reagent was required to achieve the desired thiol derivative. NEM was also found to react faster which reduces the chances of disulfide formation due to the auto oxidation of thiol. Lastly, NEM was also more effective at lower pH [41] thus it is suitable for keratin at acidic pH.

An example of maleimides (**1**) shown in Figure 5 undergoes Michael addition with a thiol group to form thiosuccinimide (**2**) and subsequently due to the instability of thiosuccinimide, it undergoes hydrolysis in biological conditions to form succinamic acid thioether (**4**). The development of maleimide bioconjugate allowed precise manipulation of cysteine side chain which is essential to the construction of therapeutics and biological probes [42].

Figure 4: Top - S-(carboxamidomethyl) keratin, Middle – S-(carboxymethyl)keratin, bottom – S-(Succinyl)keratin. Reprinted with permission from America Chemical Society [39].

Figure 5. General Michael addition and hydrolytic pathways of maleimide and thiosuccinimide. Reprinted with permission from John Wiley and Sons [42].

2.5 Keratin and Metal Binding

Removal of metal ions from an aqueous solution can be achieved in many ways via adsorption, ion exchange, precipitation, and biosorption. Adsorption uses an adsorbent to bind metal ions and this method is mostly widely used in the industry [43]. However, adsorbents such as activated carbon are costly in terms of capital and regeneration of the activated carbon. Therefore, the search for cheaper raw materials from biological origins started and led to a new term 'biosorption' where the adsorption of metal ions are achieved by using materials made from biological origin such as human hair [44]. Keratin which are found in human hair, wool, nails, horn, and epithelial covering have a distinct characteristic of having high amount of cysteine (7 to 20 number % of the total amino acid residue) [38]. Due to auto oxidation of free thiol in cysteine which form disulfide, the thiol groups are usually not available to participate in reaction as it is stable and unreactive [45, 46]. To utilize the thiol groups for coordination with metal ions, reduction of disulfide bonds were carried out [43].

Figure 6: Difference in the ease of coordination of metal ions between free thiol group and disulfide groups. Reprinted with permission from Springer Nature [43].

Since thiol is an active functional group due to the presence of sulfur, the properties of sulfur should be first understood. Sulfur is a second row element which has a coordination number between zero to seven due to their five 3d orbitals [47]. The large atomic radius of sulfur and low dissociation energy of S-H bond allow it to participate in nucleophilic activity as well as redox reactions. These activities are dependent on the ionization state of thiol which help in facilitating functions such as metal binding, nucleophilic, and redox catalysis, allosteric function and more [46]. The only amino acid with a thiol group is cysteine where the pK_a is around 8.5 and in some enzymes the pK_a of cysteine could be as low as 2.5 [46, 48]. Therefore, pH of the solution would influence the reactivity of cysteine making it more nucleophilic if the thiolate anion is stabilized in ideal conditions. Thiolate anions are formed in solutions with pH above its thiol pK_a where the rapid proton exchanges between thiols and hydroxyl groups give rise to thiolate anions. An example of the reaction is shown in Figure 7, where thiol is converted in thiolate anion and undergoes S_N2 displacement with a sulfur-sulfur bond.

Figure 7: An example of a S_N2 reaction consisting of thiolate anion. Reprinted with permission from American Chemical Society [49].

An example of the use of thiolate anions is the quantification of thiol groups using the Ellman's assay where the pH of the solution is always above pH 7 to allow formation of thiolate anions which reacts with DTNB to give a yellow colored compound. Therefore at low pHs, reactivity decreases drastically due to the protonation of the nucleophilic thiolate anion leading to decreased thiol-disulfide exchanges [48].

The importance of thiol groups is its high affinity with metal ions. For example, Cu⁺ would prefer to bind to sulfhydryl group (SH), and Cu²⁺ would prefer to bind to thiolates, ionized peptide, amines, and carboxylate [13, 46]. The solubility constant for CuS complex is K_{sp} = (6 × 10⁻³⁶) and ZnS complex is K_{sp} = (4.5 × 10⁻²⁴). This indicates that the binding of sulfur to these two metal ions are reasonably stable [13, 50]. Another concept to explain the affinity of sulfur and metal ions is the hard and soft acid and base (HSAB). Hard acids are characterized as having high oxidation state and small in size whereas soft acids are atoms with low oxidation state and large in size. Generally, hard acids would prefer to bind to hard or non-polarizable bases (N, O, F) whereas soft acids would prefer to bind to soft or polarizable bases (S, P, Cl). Stability of soft metal ion complexes for different ligand are in the order of C and S > I > Br > Cl and N > O > F [51]. Table 4 shows the examples of some elements or compounds in the different categories.

Table 4: Examples of hard soft acids in different categories. [51]

Hard acids	Soft Acids	Borderline Acids
H^+ , Na^{2+} , Mg^{2+} , Ca^{2+} , Fe^{3+} , Co^{3+} , As^{3+} , Ti^{4+} , Al^{3+}	Cu^+ , Ag^+ , Au^+ , Ti^+ , Br^+ , Cd^{2+} , Pt^{2+} , Br_2 , $Metal^0$ (metal atoms)	Cu^{2+} , Ni^{2+} , Co^{2+} , Fe^{2+} , Zn^{2+} , Pb^{2+} , $B(CH_3)_3$, SO_2 , NO^+

Following the concept, there had been synthetic resins formulated for the use in immobilized metal ion affinity chromatography (IMAC) to bind proteins which is shown in Figure 4. Ligands with cysteine and aromatic nitrogen atoms are used to coordinate with borderline acids such as Cu^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} . It is well known that histidine has the best affinity for metal ions, followed by other amino acids such as cysteine having slightly lower affinity than histidine. The affinity is mostly due to the functional groups of each amino acids such as imidazole, thiol, aromatic side chains, carboxyl groups, and phosphate groups [52, 53].

Figure 8: An example of IMAC where metal ions are incorporated to bind to targeted protein followed by desorption with a displacer. Reprinted with permission from Elsevier [53]

Table 5: Functional groups responsible for the binding and retention of protein. [53]

Functional group	Retention strength
Histidine	++++
Cysteine	++++
Aspartic acid, glutamic acid	-
Lysine, arginine	+
Tryptophan, tyrosine, phenylalanine	+
N-Terminus	++

2.6 Current Studies on Human Hair Fiber and Adsorption of Metal Ions

There are currently a few literatures on using human hair fiber for the removal of different metal ions in water. A study was done by *Tan.T.C. et al.* [54] where human hair fiber treated with an acid or base or a reducing agent was used to react with various metal ions (Ag^+ , Cu^{2+} , Ni^{2+} , Cr^{3+} , Cr^{6+} , Pb^{2+} , Hg^{2+} , Cd^{2+}). The experiment aims to find the effect of pretreatment of hair, anions, as well as the usage of single and mixed metal ions on the adsorption capacity of hair fiber. Overall, the experiment found that anion associated with the metal ions does not impact adsorption in a single metal ions system. However, in the mixed metal ions system, other metal ions could either suppress or enhance adsorption of a specified metal ion [54]. The next study done by *Amardeep Singh Saini* [55] was the removal of uranium from waste water using human hair fiber where a similar approach was applied to conduct the experiment such as varying the pH of hair fiber before treatment, effects of pretreatment of hair, and interaction time between adsorbent and adsorbate. The conclusion from the experiment was positive and human hair fibers were able to bind uranium in an alkaline condition which could possibly be used to treat uranium contaminated water [55]. Another study conducted by *Hwee Gwang Roh* [43] uses perm lotion to reduce human hair for the interaction of Pb^{2+} , $\text{Cr}_2\text{O}_7^{2-}$ and Cu^{2+} . Results of the removal of Cu^{2+} and Pb^{2+} using approximately 5 mg/ml of hair are shown in Table 6. The results show that hair can remove 88.5 % of Cu^{2+} in a 50 ppm Cu^{2+} solution and 46.5 % Cu^{2+} in a 120 ppm Cu^{2+} solution. Removal of Pb^{2+} from 20 ppm, 50 ppm and 120 ppm of Pb^{2+} solution ranges from around 90 % to 97 % as shown in Table 7. [43].

From the various literature, it can be concluded that human hair fiber can indeed bind to different heavy metals. However, many parameters such as temperature, pH, initial concentration of adsorbent/adsorbate, interaction time, and pretreatment methods can affect the efficiency and efficacy of the binding. Therefore, more studies should be conducted to understand the binding of keratin to heavy metals, starting with thiol quantification before and after interaction.

Table 6: The reduction of Cu^{2+} concentration over time with approximately 5 mg/ml of hair [43].

Initial Cu^{2+} concentration (ppm)	50	120
Removal ratio after 1 hour (%)	24	11.3
Removal ratio after 1 day (%)	73.5	32.9
Removal ratio after 2 days (%)	88.5	46.5

Table 7: Reduction of Pb^{2+} over time with different initial concentration [43].

Initial Pb^{2+} concentration (ppm)	20	50	120
Removal ratio after 1 day (%)	94.72	96.79	75.62
Removal ratio after 2 day (%)	Nil	Nil	84.98
Removal ratio after 3 days (%)	Nil	Nil	89.02

As technology gets more advanced, demands for electronics have also increased significantly, along with the increase of metal wastes generated. Therefore, it is important to manage waste via different measures such as Material Flow Analysis (MFA), Life Cycle Assessment (LCA), Extended Producer Responsibility (EPR), Advance Recycling Fee (ARF) and much more [56].

Metals found in electronic wastes can be classified into a few categories, namely [57]:

- 1) Precious metals: Au, Ag
- 2) Platinum group metals: Pd, Pt, Rh, Ir and Ru
- 3) Base metals: Cu, Al, Ni, Sn, Zn and Fe
- 4) Hazardous metals: Hg, Be, In, Pd, Cd, As and Sb
- 5) Scarce metals: Te, Ga, Se, Ta and Ge.

As the primary sources for these metals might run out eventually, the best method to manage electronic waste till date is still through Metal Recovery from E-Waste (MREW). Currently,

the few common methods used involved high temperature or strong acid, alkali, and other solvents to leech out the metals into soluble salts. Pyrometallurgical method uses physical ways to breakdown printed circuit board into smaller pieces, followed by smelting, refining, incineration, and combustion. Another method that involved high heat is the thermo-chemical method whereby thermal degradation of targeted materials were carried out. In recent years, the usage of plasma technology had been explored and deemed to be environmentally friendly. The next technique known as hydrometallurgical method uses acid, alkali, or solvent to leech metals from e-waste. After obtaining the soluble salts of the metals, the metals were recovered through electrorefining or chemical reduction [57].

One of the upcoming area of interests is biometallurgy, whereby metal was recovered using bacteria, algae, fungi etc. Although there had been some literature that showed potential in the usage of biological ligands to recover metals, the mechanisms of biosorption is complex and still being explored. In addition, there are currently no industrialized process that uses biometallurgy to recover metal [56]. Therefore, it would be interesting to explore the behaviors between extracted human hair keratin and metal binding.

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Chapter 3

Experimental Methodology

Extraction of keratin from human hair consists of many steps starting from the washing of hair fiber. In this chapter, the methods used for the extraction of keratin were broken down to understand each step better. After extraction of keratin, SDS-PAGE was used to verify the molecular weight of the proteins as well as to check on the effectiveness of the extraction procedure on the separation of proteins. Following the verification of protein, thiol quantification was carried out using Ellman's Reagent and protein concentration was determined using Bradford Assay. Thiols were also capped using NEM and quantified with Ellman's assay to ensure effectiveness of capping. Finally, the interaction between metals ions and keratin were quantified using ICP-OES.

3.1.0 Processing of Human Hair Fiber to Obtain Delipidized Hair

Random mixture of human hair was collected from various local hair salons. A portion of the hair were first washed with water and detergent followed by a second wash with 70% ethanol. The washed hair were air dried and soaked in a chloroform/methanol mixture with the ratio of (2:1 v/v) to remove the lipids in hair. The delipidized hair were air dried and cut into smaller pieces of about 2 mm.

3.1.1 Extraction of Keratin-Associated Protein (KAP) From Delipidized Hair

2.5 g of delipidized hair were soaked in 50 ml of extraction buffer consisting of 25 mM Tris HCl pH 9.5, 8 M Urea, 200 mM DTT, 25 % ethanol (v/v) and incubated at 50 °C with shaking for 72 hours. After 72 hours, the solution were filtered giving KAP as the filtrate and KAP-free hair as the residue. KAP-free hair were rinsed thoroughly with DI water to ensure KAPs were removed as much as possible followed by air drying of the KAP-free hair.

3.1.2 Extraction of Keratin From KAP-Free Hair

50 mg of KAP-free hair were soaked in 1 ml of extraction buffer consisting of 25 mM Tris HCl pH 8.5, 2.6 M Thiourea, 5 M Urea, 200 mM DTT and incubated at 50 °C with shaking for 24 hours. After 24 hours, the solution were filtered and the filtrate were collected as KIF.

3.1.3 Dialysis of Keratin/KAP against Exchange Buffer

Keratin solution were contained in a snakeskin dialysis bag with 10 kDa MWCO and dialyzed against 0.05 M citric acid pH 3.7 with 8 M Urea for 24 hours. The volume of dialysis buffer were kept at 200 times more than the sample. The same protocol was done for KAP with the exception of a 3.5 kDa snakeskin dialysis bag being used instead of 10 kDa.

3.2.0 Characterization of Keratin/KAP

To verify the molecular weight of the protein extracted, SDS-PAGE was used as it separates proteins based on their molecular weight. Having a band at 40 kDa to 65 kDa should indicate

that keratin was extracted from human hair as the keratin extraction procedure was widely used [1]. As for KAP, bands at molecular weight between 6 kDa to 30 kDa would indicate the successful extraction of KAP [1].

For the quantification of protein concentration, Bradford assay was applied. Bradford assay uses Coomassie Brilliant Blue G-250 to bind proteins which upon binding changes color from blue to red. In addition, the binding of dye to protein is a rapid process and takes about 2 minutes, thus the incubation time is reduced. Based on literature, Bradford assay is less susceptible to interferences as compared to Lowry and Biuret reaction. Bradford assay is also more sensitive than Lowry assay [2, 3].

Thiol quantification was carried out using Ellman's reagent which is 5,5'-Dithiobis(2-nitrobenzoic acid) as show in Figure 5. The reaction starts with a thiol cleaving the disulfide bond in Ellman's reagent which gives a mixed disulfide and a yellow color compound 2-nitro-5-thiobenzoic acid (TNB). The intensity of the released TNB can be measured at 412 nm and a standard curve can be constructed with a known thiol compound such as cysteine to quantify an unknown sample [4, 5].

Figure 9: Chemical structure of 5,5'-Dithiobis(2-nitrobenzoic acid) or DTNB. Reprinted with permission from American Chemical Society [5]

3.2.1 Gel electrophoresis

Gel running buffer were prepared with NuPage MES SDS Running Buffer (20x) and degas for 10 minutes. Protein samples were prepared by addition of 20 µg of crude keratin into 2 µl of sample reducing agent and 5 µl of sample buffer. The samples were heated in a water bath at 90 °C for 10 minutes. The protein samples and SeeBlue Plus 2 Pre-Stained Standard

were then loaded into NuPage 4 – 12 % Bis-Tris Gels at 150 V for 45 minutes. The gel was then removed and rinsed with DI water followed by soaking in a fixing solution consisting of 40 % ethanol and 10 % acetic acid for 15 minutes. After the protein bands were fixed onto the gel, staining was done overnight with Colloidal Coomassie Stain (Bio-Rad). Finally, DI water was used to destain the gel with slight shaking for an hour.

3.2.2 Protein Quantification

Several dilutions of keratin/KAP and BSA were prepared and concentration was determined using Bradford Assay. Briefly, 5 μ L of each dilution were added into a 96 well plate followed by addition of 250 μ L of Bradford Reagent into each well. The mixture was incubated for 5 minutes and absorbance was taken at a wavelength of 595 nm. Concentration of keratin were determined using the BSA standard curve.

3.2.3 Free Thiol Quantification

The amount of free thiol in the extracted keratin/KAP was determined using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid). Firstly, a reaction buffer consisting of 0.1 M sodium phosphate buffer, pH 8, with 1 mM EDTA was prepared. The reaction buffer was used to prepare L-Cysteine standard curve ranging from 0 mM to 1.5 mM and 10 mM Ellman's reagent stock solution. Next, 5 μ L of Ellman's reagent stock solution was added into 250 μ L of reaction buffer to give Ellman's test working reagent. For the measurement of absorbance, 25 μ L of keratin and L-cysteine were respectively added into a 96 well plate. To start the reaction, 255 μ L of Ellman's test working reagent were added into the respective samples wells and incubated for 15 minutes at room temperature. Measurement of absorbance was done at a wavelength of 412 nm.

3.2.4 Thiol Capping using NEM

Thiol capping was carried out after the amount of thiol was determined by Ellman's assay. At least 10-fold molar excess of NEM was weighed and dissolved in DI water. The excessed NEM was immediately added into the protein solution to prevent hydrolysis of maleimide

group. NEM and the protein was allowed to react for 2 hours followed by dialysis to remove excess NEM.

3.3.0 Batch Adsorption of Metal Ions (Cu (II), Ni (II), Ag (I)) and keratin

1000 ppm stock solution were prepared from the respective chloride salt for copper and nickel whereas stock solutions of silver were prepared from nitrate salt. The stock solutions were diluted into different concentration and reacted with keratin or KAP on a shaker for 24 hours. The different metal concentrations were decided based on the metal:thiol molar ratio of above 0.5 and below 0.5.

3.3.1 Batch Adsorption of Mixed Metal Ions (Cu (II), Ni (II), Al (III)) and keratin

A 1000 ppm stock solution of aluminum was prepared from its chloride salt. All three metal ions were mixed into different concentrations and reacted with keratin on a shaker for 24 hours.

3.3.2 Analysis of Metal Ions Concentration

After 24 hours, the solutions were centrifuged at 17,000 g for 20 minutes to separate any precipitate and both fractions were collected for further analysis. The supernatant was diluted with 1 % nitric acid and filtered through a 0.22 μm syringe filter prior to analysis using Inductive Coupling Plasma Optical Emission Spectroscopy (ICP-OES). A calibration curve of respective metal ion solutions were used to determine the concentration of the remaining copper ions in the supernatant.

3.4 Statistical Analysis

Quantitative values are presented as means $\pm$ standard deviation. The sample sizes for each experiment were at least three or more, unless stated otherwise. Means comparison was carried out using one-way ANOVA (for comparison between more than 2 groups) or two tailed Student's t-test (for comparison between 2 groups) where p-values of less than 0.05 indicate significant differences.

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Chapter 4

Results Chapter

The results till date will be presented in this chapter. It consists of thiol quantification of keratin in different pH buffer which shows that keratin in acidic condition had the most amount of free thiol. Next is the average yield of keratin using Shindai's extraction method for the extraction of keratin [1]. The results obtained from the extraction was 43.52 % which was comparable to the ones in literature of around 50 %. Finally, the reduction of copper ions in a solution after treatment with keratin was obtained using ICP-OES. The reduction in copper ions concentration were compared with the measured thiol concentration and a trend was observed between the two components. Once the correlation between thiol and copper ions were determined, thiol was capped with NEM. Results after capping showed no interaction between copper and keratin. Following the trend found between copper and keratin, further experiments were done with mixed metal ions and monovalent silver ions. KAP, which had higher amounts of cysteine, was also reacted with metal ions and results showed better interaction for KAP compared to keratin.

4.1 Keratin Extraction Using Shindai's Extraction Method

There are two major proteins in human hair, namely keratin and keratin-associated proteins (KAPs). The molecular mass of keratin are 40 - 65 kDa and molecular mass of KAPs are 6 - 30 kDa [1]. Keratin can be further classified as Type I (acidic) and Type II (basic) monomers with molecular mass of around 45 kDa and 50 kDa respectively [2]. From Figure 10, the extracted proteins had thick bands in the region between 38 kDa to 62 kDa. This shows that the extraction was successful and keratin was present. Other bands were also observed at around 98 kDa and 28 kDa. The higher molecular mass shows that there would most likely be keratin dimers present and the lower molecular mass suggested that some KAPs were also present after the extraction.

Figure 10: Bands showing the different molecular weight proteins present in the sample with the most prominent bands from keratin and a faint bands of KAPs in the lower molecular weight region.

4.2 Keratin Concentration and Free Thiol Quantification

50 mg of KAP-free hair were used with 1 ml of keratin extraction buffer for the extraction of keratin. After extraction, keratin was dialyzed in different buffers with various pH of 3.7, 4.5, 7.5 and 9.5. From Figure 11, at pH 4.5, gel was formed as the pH range is within the isoelectric point of keratin. For pH 7.5 and 9.5, the keratin exhibited some gelling as seen from Figure 12, suggesting that some thiol group might have crosslinked into disulfide which

would affect the study between thiol and copper interaction. In addition, pipetting of gel might not be accurate as it would stick to the side of the pipette tip. Therefore, keratin in acidic pH was selected for future studies.

Figure 11: Appearance of keratin in different pH buffers.

Figure 12: Inversion of keratin solution to demonstrate the gelling of keratin in pH 7.5 and 9.5 whereas at pH 3.5 KIF was able to flow downwards.

The dialyzed keratin was quantified by Bradford Assay to determine their concentration. Table 8 shows the protein concentration of various keratin samples and an average yield of 43.52 % were calculated. Figure 13 depicts the average protein concentration and the standard deviations from 17 batches of keratin. Using one-way ANOVA test, p-value of > 0.05 were obtained which shows that the difference in protein concentration between batches were not statistically significant. The yield obtained were comparable to [1] where 1 g of hair sample gave a yield of 510 mg of keratin.

Table 8: Protein concentration of various keratin in citric buffer, 8 M urea. Yield of keratin were calculated based on 50 mg/ml of initial concentration.

Sample	keratin concentration (mg/ml)	Yield (%)	Average Yield (%)
1	20.35	40.70	43.52
2	14.57	29.14	
3	21.68	43.36	
4	25.71	51.42	
5	19.25	38.50	
6	30.66	61.32	
7	26.79	53.58	
8	24.36	48.72	
9	21.35	42.70	
10	19.92	39.84	
11	18.87	37.74	
12	26.04	52.08	
13	22.75	45.50	
14	21.31	42.62	
15	20.55	41.10	
16	18.63	37.26	
17	17.13	34.26

Figure 13: Average keratin concentration obtained from 17 batches of extraction.

(n = 17, p > 0.05, ANOVA)

After extraction of keratin, free thiol quantification using Ellman's assay was carried out. Generally, a higher amount of free thiol is desired as it is more reactive which would allow for more reactions and chemical modifications of keratin. Table 9 shows the free thiol concentration in various samples as well as the concentration after normalizing with its protein concentration. Since different batches of extracted keratin would have different amount of free thiol and protein concentration, the normalized free thiol concentration would be a better measurement for comparison between different batches. Figure 14 shows the average normalized thiol concentration and the standard deviations from 17 batches of keratin. Using one-way ANOVA test, a p-value of > 0.05 was obtained which suggested that the difference in the normalized thiol concentration was not significant.

Table 9: Free thiol concentration determined by Ellman assay and the thiol concentration that was normalized by individual protein concentration.

Sample	Thiol concentration (mM)	Normalized thiol concentration (mmol/g)	Average thiol concentration (mmol/g)
1	18.91	0.93	1.06
2	18.95	1.30	
3	17.74	0.82	
4	19.35	0.75	
5	32.21	1.68	
6	48.78	1.59	
7	14.01	0.52	
8	17.62	0.72	
9	19.35	0.91	
10	16.31	0.82	
11	26.825	1.42	
12	23.22	0.89	
13	27.2	1.20	
14	21.9	1.03	
15	26.5	1.29	
16	18.5	0.99	
17	20.0	1.17

Figure 14: Average of the normalized thiol concentration between 17 batches of keratin. (n= 17, p = >0.05, ANOVA)

4.3 Interaction between Free Thiol and Copper ions

Table 10: Initial copper concentration used based on thiol concentration to keep molar ratio of copper thiol above and below 0.5.

Sample No.	KIF Thiol concentration (mmol)	Molar ratio of $\frac{[Cu]}{[Thiol]}$									
		Copper initial concentration (mmol)									
		0.00134	0.00268	0.00357	0.00534	0.00625	0.00803	0.00893	0.01071	0.0125	0.01339
1	0.040			0.13				0.27			0.53
2	0.038			0.14				0.28			0.56
3	0.013			0.43				0.85			1.71
4	0.017	0.08	0.15	0.31				0.62			
5	0.011		0.25	0.33	0.49		0.74	0.82			
6	0.016		0.16	0.22	0.33		0.49	0.55			
7	0.017		0.16		0.31			0.52	0.63	0.73	0.79
8	0.011		0.25	0.33		0.58	0.75	0.83			
9	0.014		0.19	0.25	0.37		0.56	0.62	0.74		
10	0.014		0.20	0.26	0.39		0.59				
11	0.016		0.16						0.65	0.76	
12	0.015		0.17	0.23				0.58	0.69

Figure 15: Reduction of copper concentration from solution with varying thiol concentration.

Figure 16: The reduction of Cu concentration with different ratio of $[Cu]/[Thiol]$ concentration showing a few samples with molar ratio > 0.5 where there is unexpected reduction of Cu concentration.

In the experiment procedure, copper solutions were prepared based on the amount of thiol present in keratin. From literature, 2 moles of thiol would react with 1 mole of Copper (II) to produce a thiol-copper (I) complex, disulfide and hydrogen ions as shown in equation (6) below [3]. A few researchers had also conducted experiments with different thiol compounds and found that at a copper:thiol mole ratio of more than 0.5, disulfide would be formed instead of thiol-copper (I) complex, while a ratio of less than 0.5 will promote formation of the thiol-copper (I) complex. [4-6]

The experimental parameters are shown in Table 10, where different concentrations of copper solutions were used to provide a range of copper:thiol molar ratio of either below or above 0.5. The reduction of copper concentration in relation to the molar ratio are shown in Figure 15 where about 80 % reduction in copper concentration were observed when the molar ratio is below 0.5. This result is aligned with the results observed from [4-6] where thiol-copper (I) was formed and subsequently removed from the solution as precipitates leading to the reduction of copper ions in the remaining solution. For copper:thiol molar ratio of above 0.5, reduce in copper concentration of around 10 % was observed. Based on experimental results conducted by the authors in literature, an excess of copper ions would lead to thiol-copper (I) complex being converted to disulfide and the release of copper back into the solution as shown in equation (11). Therefore, removal of copper was low as the thiol complex is oxidized back into an unreactive disulfide. A possible reason for the oxidation of thiol into disulfide could be due to the generation of reactive oxygen species in aerobic conditions which was catalyzed by Cu (II). However, some authors had also observed such phenomenon in an anaerobic condition. Therefore, the oxidation of thiol catalyzed by Cu (II) requires further investigation to fully understand the redox mechanisms involved.

The low amount of binding could be due to amine or carboxyl functional groups which could also bind copper. In addition, keratin has low amount of histidine (about 1 %), tryptophan (about 0.4 %) and phenylalanine (about 2 %) which could also bind to metal ions. However, the retention of metals are not as strong compared to histidine and cysteine [7, 8]. In addition, the low amount of binding and large standard deviations could be due to experimental variations from different batches of samples. As shown in Figure 16, there was unexpected copper concentration reduction in some samples with molar ratios > 0.5 . This could be due to inaccuracies and variations in thiol or copper concentrations, which will be addressed with larger sample sizes to be carried out in the future.

4.4 Batch Adsorption of Copper Ions and Keratin

Figure 17: Initial concentration of copper ions (left bar) and the remaining copper ions (right bar) after interaction with keratin. Values are represented as mean $\pm$ SD, $n = 4$, (60 ppm, 80 ppm: $*p < 0.05$ vs initial concentration), (120 ppm, 180 ppm, 200 ppm, 240 ppm: not significant (NS) $p > 0.05$ vs initial concentration), two-tailed t test.

Batch adsorption of copper with keratin was done by varying the initial concentration of copper solution and keeping keratin at 2 mg/ml. Figure 17 shows the initial concentration of copper and the amount left in the solution after interaction with keratin. From the chart, copper ions concentration at 60 ppm and 80 ppm decreased significantly after interaction

with keratin. The reduction for both concentrations were about 80 % and there were significant differences between the experimental results and initial concentration. Due to the high copper ions concentration above 180 ppm, there were no significant reduction in copper concentration observed which was likely due to the oxidation of free thiol groups. At 120 ppm, although results from two-tailed t-test showed that reduction of copper ions concentration in the solution was not significant, the reduction was about 50 % with large standard deviations. This is because at 120 ppm, the molar ratio between copper and thiol is always near 0.5, therefore, reduction of copper fluctuates due to the uncertainty between oxidation of thiol groups and binding of metal ions.

4.5 Thiol Capping of keratin and its Effect on Copper Ions Binding

Figure 18: Keratin adsorption of Cu ions before and after thiol capping. Values are represented as mean $\pm$ SD, $n = 3$, before capping: * $p < 0.05$ vs initial concentration, after capping: not significant (NS) $p > 0.05$ vs initial concentration, two-tailed t test.

To validate the hypotheses that thiol groups in keratin were the main functional group responsible for the interaction between keratin and copper ions, thiol in keratin were capped with NEM. The remaining copper ions left in the solution were measured after interaction

with the capped and non-capped keratin respectively. In Figure 18, the middle blue bar shows the final concentration of copper ions in the solution after thiol groups in the keratin were capped with NEM and the green bar on the right shows the copper concentration with non-capped keratin. The results from the non-capped keratin were similar to previous results as shown in Figure 17 whereas the capped keratin had no significant reduction. In addition, two-tailed t-test results showed that copper concentration reduction before keratin capping was significantly different when compared to the initial concentration, while the opposite is observed for capped keratin. This proves that thiol groups were indeed the main functional group responsible for the binding of copper ions.

4.6 Batch Adsorption of keratin with Another Metal⁺² Ion (Ni²⁺)

Figure 19. Initial concentration of nickel ions (left bar) and the remaining nickel ions (right bar) after interaction with keratin. Values are represented as mean $\pm$ SD, $n = 3$, 60 ppm $*p < 0.05$ vs initial concentration, (80 ppm, 180 pm: not significant (NS) $p > 0.05$ vs initial concentration), two-tailed t test.

The use of nickel (II) to interact with keratin was to determine the trend between keratin and

M^{2+} ions. Since copper (II) showed a certain trend when interacting with keratin and nickel is located just beside copper, it was interesting to find out if nickel (II) had similar interaction with keratin as compared to copper (II). Following the copper (II):thiol molar ratio concept of above 0.5 and below 0.5, nickel (II) concentration at 60 ppm and 80 ppm had a molar ratio of below 0.5 whereas at 180 ppm, it had a molar ratio of above 0.5. Based on the previous finding using copper (II) and keratin, there should be significant reduction in concentration when the molar ratio is below 0.5. Although t-test results showed significant differences for Ni concentration at 60 ppm, the reduction was only about 9 %. From Figure 19, it was observed that even though both 60 ppm and 80 ppm nickel solution had a copper:thiol molar ratio of < 0.5 , there were no significant reduction in concentration after it interacted with keratin. This might suggest that keratin is selective in the metal ions that it binds to even though there are presence of free thiol groups. Based on the Irving William series, the least stable metal ligand complexes starts from: $Mn < Fe < Co < Ni < Cu > Zn$ [9] where Ni is able to form relatively stable complexes with ligands. In an article by *Steven A. Ross and Cynthia J. Burrows*, the authors synthesized different peptides with cysteines that contained either free thiol, an S-tert-butyl-protected thiol or dimer consisting of disulfide. The compounds were reacted with Ni (II) and the products were characterized. Findings from their experiments showed the peptide synthesized with free thiol groups will always be oxidized by Ni (II) under aerobic conditions resulting in the formation of disulfide bonds [10]. Other authors, *Masri, M.S. and Friedman, M*, treated wool under different conditions and found that wool that were reduced (WSH) had significant uptake of copper (I) and negligible nickel (II) uptake. The article also suggested that copper (II) had poorer uptake as compared to copper (I) and both copper (II) as well as nickel (II) could have oxidized the free thiol group into disulfide which resulted in the poor uptake of both metal ions [11].

4.7 The Effects of Mixed Ni^{2+} and Cu^{2+} Solution on the Adsorption Capacity of Keratin

Figure 20. Initial and final concentration of Nickel and Copper ions after interaction with keratin. Values are represented as mean $\pm$ SD, $n = 3$, (Cu 60, 80 ppm * $p < 0.05$ vs initial concentration, Cu 180 ppm (not significant (NS) $p > 0.005$ vs initial concentration), (Ni 60, 80, 180 ppm, (NS) $p > 0.05$ vs initial concentration), two-tailed t test.

Binding of metal ions to peptide can be easily affected by the presence of another competing metal ion. Other parameters such as the pH, configuration of the peptide upon metal binding, stability of the complex formed and many more could affect metal peptide binding. [8, 12]. In this section, nickel ions were mixed with copper ions and interacted with keratin to determine the effects it had on the binding of copper ions to keratin. The number of moles of copper and nickel ions were calculated and the molar ratio between both metal ions and thiol were kept below and above 0.5. This was to determine if the combined moles of M^{2+} ions:thiol would have similar results as compared to only copper (II) ions. From the calculation, at 60 ppm and 80 ppm of mixed nickel and copper concentrations, there should be significant decrease of the metal ions. T-test results showed that at 180 ppm, both results are not significantly different as compared to the initial concentration. This was expected as the molar ratio between copper and thiol is above 0.5 which would mean the free thiol groups

will be oxidized into disulfides. The results obtained in Figure 20 showed that the total molar ratio of the mixed M^{2+} ions did not affect the ability of keratin to reduce the concentration of copper ions in the solution. This indicates that the reaction as shown in equation (6), $2RSH + Cu(II) \rightarrow RS-Cu(I) + 0.5 RSSR + H^+$ is the dominant reaction.

One of the possible reasons might be due to the stability of Cu^+ ions as compared to Ni^+ ions. The most common oxidation state of nickel is Ni (II) followed by Ni (0) whereby both Ni (0) and Ni (II) often participate together in catalytic reactions. Some of the catalytic reactions were thought to involve Ni (I) and Ni (III) oxidation states but Ni (I) complexes are unstable, which leads to the difficulty of studying it as a variety of ligands are required to stabilize it [13]. An article in 2018 also indicated that Ni (I) was proposed to participate in many catalytic reactions, however the fundamental studies on the reactivity and stability of Ni (I) species was still lacking. In the article, the authors synthesized a series of Ni (I) aryl species and suggested that Ni (I) aryl species were unstable compounds which decomposed to form Ni (0) [14]. Therefore, Ni (I) complex is rare and this might be due to the electronic configuration of Ni which is $[Ar] 3d^8 4s^2$ where there are 2 paired electrons in the 4s shell. In comparison, Cu has an electronic configuration of $[Ar] 3d^{10} 4s^1$ where there is only 1 electron in the 4s shell which makes it easier to be donated as compared to a paired electron. Lastly, it could be possible that Cu was able to form a stable structure such as a tetrahedral complex after coordinating with keratin which Ni was not able to achieve. However, these suggestions have to be tested and prove its validity. Thus, Cu (I) are more readily formed as compared to Ni (I) which might explain the selectivity of keratin to bind Cu instead of Ni.

4.8 Batch Adsorption of Mixed Metal Ions with keratin

Figure 21: Keratin metal adsorption capability in a mixed metal ions solution containing M^{2+} , M^{3+} . Values are represented as mean $\pm$ SD, $n = 4$, (Ni, Al 60 ppm NS $p > 0.05$ vs initial concentration, Cu 60 ppm * $p < 0.005$ vs initial concentration), (Ni, Al, Cu 180 ppm, (NS) $p > 0.05$ vs initial concentration), two-tailed t test.

Following the previous experiment with two mixed metal ions, an experiment consisting of three mixed metal ions and keratin were carried out to find out the effects they had on keratin metal binding. The mixture consists of two M^{2+} (Ni^{2+} , Cu^{2+}) and one M^{3+} (Al^{3+}) ions. Figure 21 shows the concentration of the remaining metal ions in the solution after interaction with KIF. The final concentration of copper at 60 ppm showed similar reduction amount as compared to the single and two mixed metal ions system. Nickel had negligible decrease in concentration which was expected as discussed previously in Sections 4.4 and 4.6. Aluminum also had negligible reduction in concentration which was expected as well. As Al^{3+} is a hard acid due to its small ionic radius and high positive charge, it is expected to bind well to hard base instead of soft base like sulfur. The result from the experiments above

suggests that keratin is highly selective towards copper between the few metal ions selected and it is not easily displaced by nickel or aluminum.

The results obtained from the above experiments agree with other published papers where keratin fibers were used for the interaction with metal ions. One of the authors used different keratin fibers (human hair, dog hair, degreased wool, and chicken feather) to interact with a mixture of heavy metals consisting of Cr (III), Mn (III), Co (II), Ni (II), Cu (II), Zn (II), Cd (II) and Pb (II). From the results in Figure 22, it could be seen that similar results were obtained for human hair fiber where Cu had significant adsorption to human hair and Ni had negligible adsorption [15].

Figure 22. Biosorption of human hair, dog hair, chicken feathers, and degreased wool for Cr (III), Mn (III), Co (II), Ni (II), Cu (II), Zn (II), Cd (II) and Pb (II) in multiple-metal aqueous system. Initial metal concentration 0.1 mmol of each metal ion/L, contact time 24 h, initial pH 4.0, and 0.1 g of biosorbent in 10 ml of initial solution. Reprinted with permission from SAGE publication [15].

Another study by *Tan.T.C. et al.* [16] used pretreated human hair fiber to react with a series of mixed metal ions and found that different metal ions might promote or suppress the adsorption of each individual metal ions. From their results, the authors concluded that

affinity of metal binding to their pretreated human hair fiber are in decreasing order from $\text{Hg}^{2+} > \text{Ag}^+ > (\text{Cu}^{2+}, \text{Pb}^{2+}, \text{Cr}^{3+}) > \text{Cd}^{2+} > \text{Ni}^{2+}$.

4.9 Batch Adsorption of Silver Ions with Keratin

Figure 23: Keratin adsorption of monovalent metal ion (Ag^+). Values are represented as mean $\pm$ SD, $n = 2$, $p < 0.05$ vs initial concentration), two-tailed t test.

Following the HSAB concept by Pearson, soft acids such as Ag^+ would prefer to bind to soft base such as sulfur [17]. It was also well known that silver has antimicrobial properties that is closely linked to their interaction with thiol groups. Amino acids such as cysteine and other compounds containing thiol had been found to neutralize the antibacterial activity of silver while compounds with disulfide did not [18]. Another study had similar results and reported the coordination number between silver and cysteine or reduced glutathione to be 3:1. These shows that thiol-containing compounds were essential in the binding of Ag^+ ions [19].

In this experiment, the functionality of thiol in keratin to bind Ag^+ ions was evaluated. Unlike

copper (II) ions which requires a copper:thiol ratio of below 0.5 to form RSCu(I) compounds, silver ions can have a higher ratio of 3 silver ions:1 thiol. This would allow keratin to bind to silver at a much higher concentration as compared to copper. This observation can be seen from Figure 23, where silver ions were removed from the solution even at high concentrations. Comparing this result with Figure 17, the concentration of silver ions was twice as much as the concentration of copper ions and keratin was still able to interact with the silver ions resulting in the large decrease. These results were expected as it was well known that silver had strong affinity with thiol and the results showed that thiol in keratin had similar interaction with silver as other thiol compounds.

4.10 Batch Adsorption of Copper Ions and KAP Before and After Thiol Capping

Figure 24: Initial and final concentration of copper ions after interaction with KAP. Values are represented as mean $\pm$ SD, $n = 3$, (Cu 80, 120, 240 ppm * $p < 0.05$ vs initial concentration), Cu 280 ppm not significant (NS) $p > 0.005$ vs initial concentration, two-tailed t test.

One of the differences between keratin and KAP is their cystine content, keratin consists of about 6 % of cystine whereas KAP has about 21 % of cystine which is approximately 3.5 times more [2]. Therefore, a lower protein concentration from KAP was required to achieve the copper:thiol molar ratio of less than 0.5 and more than 0.5. The green bar on the right in Figure 24 shows the final concentration of copper ions after interaction with KAP. Based on the previous observation from keratin, it was expected that there would be significant reduction from 80 ppm to 120 ppm as the molar ratio between copper and thiol is below 0.5 in these concentration range. However, KAP was also able to cause some reduction of copper even in the range of 240 ppm to 280 ppm which had copper thiol molar ratio above 0.5. These results were not expected as the free thiol in that range should have been oxidized into disulfide which would lead to the non-binding of copper ions to KAP. Therefore, KAP might have other characteristics which allowed it to bind to copper ions even at high concentrations. Although the high concentration of copper ions did not prevent the binding of copper to KAP as expected, capping of the thiol in KAP prevents the binding of copper to it. This would indicate that thiol contributed greatly to the metal binding behavior of KAP, which once inhibited would prevent KAP from binding to copper.

4.11 Comparison of Copper Adsorption between Keratin and KAP

Figure 25. Normalized adsorption of copper ions in per gram of Keratin and KAP. Values are represented as mean $\pm$ SD, $n = 3$, KIF adsorption vs KAP adsorption (* $p < 0.05$), one-tailed t test.

As the thiol concentration between keratin and KAP differs greatly, the initial protein concentration used were also different as to achieve the desired copper:thiol molar ratio. To make a fair comparison between the adsorption capacity of keratin and KAP, the results were normalized against the protein concentration of keratin and KAP respectively. Since the cystine content of KAP was approximately 3.5 times more than keratin, the adsorption was also greatly increased as shown in Figure 25.

4.12 Comparison of Copper Adsorption between Human Hair Fiber in Literature and Extracted Hair Keratin

Figure 26: Comparison between the adsorption of copper ions in human hair fiber from literature and the calculated KIF.

Figure 27: Comparison between literature and experimental data on the absorption of copper ions with KIF.

With the extracted KIF, it is assumed that functional groups involved in binding of metal ions are readily exposed, thus it should bind more efficiently to metal ions. In comparison, in human hair fibers, metal ions need to penetrate through pores and cracks in the hair surface

to get adsorbed, and surface-exposed functional groups involved in metal ions binding are limited. A few reports in the literature [20, 21] used hair treated in different conditions to interact with copper ions and results at low initial copper concentrations were promising, showing a reduction of about 80%. Figure 26 compares the adsorption of copper ions onto human hair fibers and the calculated theoretical keratin amounts based on the experimental keratin yield of 43.52% as shown in Table 8. As expected, the extracted keratin should theoretically have better adsorption compared to hair fiber. Finally, in Figure 27, the calculated keratin amounts from literature were compared with experimental keratin and it showed that even at higher copper concentration, the experimental keratin were able to adsorb more copper ions than the ones reported in the literature. Therefore, extracted keratin will have better efficacy than human hair fibers in the adsorption of metal ions.

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Chapter 5

Conclusion and Recommendations

In biology, proteins containing sulfur could act as a monodentate or bidentate ligand which could bind to many metals leading to a variety of coordination chemistry [1]. Therefore, more studies could be carried out to find out the kinetics and thermodynamics of this metal-keratin interaction. Different environmental conditions of the protein could also result in activation of different functional groups or physical properties of keratin. Hence, it would also be interesting to find out the interaction between metals and the respective functional groups in keratin. In addition, since keratin is able to form fibrous network, it would also be beneficial to understand the formation of these networks in the presence of metal ions so as to possibly bind metal ions through chemisorption and physisorption.

5.0 Conclusion

With the rising demand of electronics, more and more research had been done to recover metals from electronic waste. As more and more bio-sorbents are explored, it is also important to look deeper into their reaction mechanisms. In this thesis, keratin and keratin associated proteins were successfully extracted from human hair using Shindai's method. The formation of disulfides or thiol-copper complexation in keratin was found to be driven by the molar ratio between copper and thiol. Copper thiol molar ratio below 0.5 would led to the formation of thiol-copper complex whereas molar ratio above 0.5 would led to formation of disulfides.

From the experiments, it was found that 2 mg/ml of keratin was able to achieve 80% to 90% reduction of copper ions from a solution that was up to 120 ppm. These reductions were not observed once the thiols were capped with NEM. This further shows the importance of thiols in keratin for the interaction with metal ions. Between silver, copper, nickel and aluminum ions, silver monovalent ions had the highest adsorption to keratin whereas trivalent aluminum had the least adsorption. In a three mixed metal solution, nickel and aluminum did not affect the adsorption of copper to keratin.

Between keratin and KAP, KAP was about 3.5 times more effective in binding metal ions due to the higher amounts of cysteine in KAP. This further proved the hypothesis that thiols in keratin remains functional and are the main functional groups responsible for keratin mediated reduction of metal ions. Finally, a comparison between human hair fiber and extracted keratins showed that the latter is significantly more efficient in reduction of metal ions. In conclusion, hair extracted keratin has the potential to be exploited as a substrate for chelating and harvesting metals from a solution of metal ions. This approach also achieves recycling of human hair, which is a significant source of bio-waste.

5.1 Recommendations

5.1.1 Adsorption isotherm and thermodynamic studies

Since the preliminary study on the binding of keratin to copper ions showed favorable results, a deeper understand on the enthalpy, entropy and Gibbs free energy of the reaction could be done. Apart from copper ions, other metal ions could be tested with keratin to determine their compatibility. One of the method could be with the use of an isothermal titration calorimeter where heat absorbed or released due to the mixing of samples and reactants are captured by the calorimeter. This information can be further analyzed to provide the binding stoichiometry between the ligand and reactant as well as the mode of binding. There are two popular isotherm to evaluate the adsorption capability of an adsorbent. Experimental data are fitted into the isotherms model and the best fitting isotherm would be used to explain the binding results. The first isotherm is known as Langmuir adsorption isotherm which depends on the affinity between solutes and adsorbents to determine the maximum adsorption capacity per mass unit of adsorbent. Langmuir isotherm is suitable for smooth, flat single layered surfaces. However, one of the disadvantages of this isotherm is that it does not account for adsorbate-adsorbate interaction. The next isotherm is known as Freundlich adsorption isotherm. Unlike, Langmuir adsorption isotherm, Freundlich adsorption isotherm measures the increasing amount of adsorbates per mass unit of adsorbents over time. The increased of adsorbates were due to the stacking of adsorbate molecules, therefore, adsorbate-adsorbate interaction was taken into consideration. One of the disadvantages of Freundlich isotherm is that it is empirical and only provides an approximation of the adsorption behaviors, hence it cannot be used to generalize different adsorption systems.

5.1.2 Systematic studies on the effect of pH on metal binding

The role of pH in the solution has the ability to affect many parameters such as metal stability, nucleophilicity of the ligand, electron-donor acceptor properties of the solutes [2]. To coordinate to a protein's binding sites, heavy metal ions have to compete with protons. Therefore, adsorption of metal on keratin should increase with pH as more thiol groups would be deprotonated. However, the isoelectric point (pI) of keratin ranges from 4.5 to 7

[3] which upon reaching this pH, keratin would be precipitated. In addition, most metal would precipitate at higher pH of about 6-7 due to the formation of metal hydroxide. Although a higher pH above the pI of keratin would result in more thiolate anions which should increase the efficiency of metal ions binding, it would also cause more rapid formation of disulfides. Hence, a systematic study on the effects of pH on keratin metal binding would provide more insights into the relationship between thiol of keratin and metal binding under different pH conditions. Furthermore, as pH changes, the physical properties of keratin would change as well such as the formation of gels, precipitates or remain aqueous.

5.1.3 Formulation and characterization of keratin metal complex

Keratin are intermediate filament which form fibrous networks due to the various interaction such as hydrogen bonds, hydrophobic interaction, disulfides bond and ionic bonds [3, 4]. Further manipulation of the disulfides and environmental conditions of keratin could be explored to formulate keratin gels or film for the use of electronic waste recovery. A balance between free thiol groups and disulfides could lead to a keratin fibrous compound that is capable of both physical and chemical interactions of metal ions. Under different environmental conditions, others amino acids functional groups could also be activated to interact with different metal ions to form a stable configuration. As keratin could polymerize by itself to form fibrous structure, it may be subjected to various parameters such as pH, ionic strength of buffer, temperature, duration to achieve different degree of polymerization. Alternatively, keratin and keratin associated proteins could be mixed in different ratio to achieve different degree of polymerization which will result in a solid complex with a wide range of mechanical and chemical properties. Therefore, to make keratin feasible to be used for applications, a physical compounds should be formed. Thus, different formulation of keratin complexes should be explored. Apart from fabrication of keratin compounds, characterization has to be done as well to understand the changes occurring in the compounds. Analysis of the functional groups before and after a reaction can be done by a few methods such as Fourier-transform infrared spectroscopy, Ramen spectroscopy or ultraviolet-visible spectroscopy. Physical appearance of the keratin complex can be seen from scanning electron microscopy or transmission electron microscopy. Lastly, mechanical properties

such as tensile strength, modulus, elasticity, etc can be analyzed by a range of mechanical tester.

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