CHAPTER 1

INTRODUCTION

1.1 LEATHER A UNIQUE MATERIAL

The skin of animals has several distinctly varying functionalities from maintenance of body temperature to serving as a protective layer in playing the role of excretion of cell wastes. To be able to perform these varying functions; animal skins are structured in unique manner. The animal skin is unique in terms of its functionalities, structure and chemistry. Leather is one of the earliest materials of mankind and used as a material for apparel. In the present day, leather is utilized for as many as 200 applications. Despite, the advent of many advanced and inexpensive materials as substitutes, leather cannot be replaced completely. Uniquenes of leather is primarily inherited from the raw material i.e skin or hide and the science and technology of manufacturing processes leading to several products from the same raw material.

1.1.1 Leather – Life Cycle Thinking

Almost 0.72 million kg of leather is disposed every year globally. In the process of conversion of animal skin into leather, the former, which is susceptible to putrefaction, is stabilized. During the pretanning processes, the animal skin is primarily prepared and conditioned to render it suitable for the second and important step of tanning. During tanning, the collagen, the leather making protein is crosslinked. The kind of linkage, degree of
stabilization, physical properties that could be rendered vary with various tanning agents and systems. The ‘tanned’ leather is made significantly resistant to the enzymes secreted by organisms of putrefaction of hide and skin. Tanned material also acquires certain characteristics that are amenable to physical and chemical forces. Hence, the biodegradability of the animal skin is significantly reduced by tanning. Whereas, this is the imminent to use leather for making various desired articles and products, it is a concern when it comes to disposal of the leather products. In the holistic life cycle analysis of safe disposal, the biodegradability of leather gains immense importance. There are two fundamental strategies for addressing the above issue. Either a process of degradation of leather without significant environmental impact need to be developed or a process of tanning which could render leather relatively amenable for biodegradability needs to be developed. In the second strategy, there exists a conflict. While intending to make biodegradable leather, the fundamental requirement of rendering resistance to the attack of micro-organism shall not be compromised. Development of process for the manufacture shall primarily aim at identification of tanning processes imparting ‘leather’ characteristics to hide or skin and at the same time not losing the biodegradability of tanned leather significantly on disposal under certain environmental conditions.

1.2 TANNING – AN OVERVIEW

In depth and thorough understanding of tanning is essential to address the issue and development of tanning system that can render biodegradable leather. Tanning systems are classified on the nature of the tanning material used as (a) Inorganic tanning systems and (b) Organic tanning systems (Gustavson 1956).
1.2.1 An Overview of Inorganic Tanning Systems

The commonly used inorganic tanning agents based on elements like chromium, iron, aluminum, titanium and zirconium and used in the leather industry (Chakravorty and Nursten 1958) are given in Table 1.1. Amongst all, chrome tanning is a well-known tanning system because of high shrinkage temperature and tensile strength, it imparts to leather.

Table 1.1 Comparison of Mineral Tanning Agents

<table>
<thead>
<tr>
<th>Properties</th>
<th>Cr(III)</th>
<th>Fe(III)</th>
<th>Al(III)</th>
<th>Ti(IV)</th>
<th>Zr(IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum $T_s^*$</td>
<td>120</td>
<td>85</td>
<td>88</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>Tanned Leather characteristics</td>
<td>Wet blue</td>
<td>Brown</td>
<td>White</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leather</td>
<td>leather</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Shrinkage Temperature

Basic chromium sulfate (BCS) is used as the conventional common chrome tanning agent in the leather industry. It has the ability to interact with aspartic and glutamic acid at pH 4 to form coordinate covalent bond (Shuttleworth 1950, 1952). BCS in aqueous solution results in more than 14 different species (Chandrasekaran et al 1999). The extent of hydrothermal and enzymatic stability of collagen is dependent on the molecular structure of the bound Cr(III) species (Covington 1997, Gayathri et al 1999, 2000, 2001). The mechanism of iron tanning is considered to be similar to that of chrome tanning (Tavani and Lacour 1994). An iron salt seems to be an alternative tanning agent to chrome, which exhibits shrinkage temperature greater than 85 °C (Balasubramanian and Gayathri 1997 and Thanikaivelan et al 2000).

Aluminium(III) in contrast to chromium, does not form stable coordination complexes because aluminium forms highly labile complexes
and the rate of exchange of solvate ligands of Al(III) is $10^9$ times faster than that of chromium (Covington 1997). The aqueous chemistry of titanium(IV) is dominated by the titanyl ion TiO$_2^+$ (McAulife and Basratt 1987) and Ti(IV) is a $d^0$ ion with limited stability of the ligand fields. Potassium titanyl oxalate is used for tanning (Swamy et al 1983) and these systems were more successful in retanning of vegetable tanned leather (Ramasami 1994). Zirconium(IV) is also a $d^0$ system and coordination geometry of Zr(IV) in aqueous system is characterized by eight coordination geometry and oligomeric structures in which tetrameric complexes form basic building blocks (Ranganathan and Reed 1958, Hock 1974). The use of basic zirconium sulfate as a self tanning material is limited because of low hydrothermal stability and high acidity conditions needed for tanning that leads to weak leather with drawn grain at higher cost compared to chrome tanning. However, more efficient zirconium tanning agents have been developed (Sreeram et al 2000).

1.2.2 An Overview of Organic Tanning Systems

Tanning systems which are associated with tanning materials of organic nature are called as organic tannages. These include oil tannins, aldehyde tannins and vegetable tannins.

Oil tanning is one of the ancient tanning materials. Fish oil tanning is a popular tanning method employed specifically for chamois leathers. The mechanism of oil tanning is similar to that of the aldehyde tanning. Vegetable tannins containing polyphenols of hydrolysable (gallo tannins and ellagi tannins) and condensed type tannins with molecular weight in the range of 500-3000 are well established tannins for leather processing (Atkinson 1993). The universally accepted mechanism for vegetable tannage is through hydrogen bonding to the CO-NH linkage of the protein and the phenolic
hydroxyl group of the vegetable tannins. Depending on the pH conditions, polyphenols also fix to amino side chains by electrostatic salt links with carboxylate or hydrogen bonding with carboxylic acid groups.

The tanning of skin with mono and difunctional aldehydes such as formaldehyde, glyoxal and glutaraldehyde were well reviewed (Wojdasiewicz et al 1992). Aldehydes react with proteins and harden them, resisting from putrefaction and decomposition and the treated collagen behaves like leather and therefore these aldehydes are used in tanning industry as a tanning materials. Among the monoaldehydes and dialdehydes known, formaldehyde, glyoxal and glutaraldehyde exhibit good tanning properties and the unsaturated aldehydes like acrolein, crotonaldehyde result in fair degree of tanning (Gustavson 1956). Though, aldehydes react with collagen practically in all pH ranges i.e. acidic, neutral and alkaline; in practice, tanning is done in slightly alkaline medium as the fixation of aldehyde to collagen increases with increase in pH. The fixation of aldehyde to collagen depends mainly on two factors such as pH and concentration of aldehyde in solution. At higher pH values and concentration, aldehyde fixed to collagen is higher. The amino groups present in collagen are involved in interaction with formaldehyde and crosslinking with dialdehydes (Bowes and Cater 1968). Formaldehyde is known to interact with the amino groups of protein, which is followed by further condensation with amides and guanidyl groups.

An earlier study on the treatment of collagen with glyoxal results in decrease of lysine and hydroxylysine, suggesting that the bond formed is relatively stable (Bowes and Cater 1968). Glutaraldehyde reacts with the α-amino groups, N-terminal amino groups of peptides and the sulfhydryl group of cysteine (Habeeb and Hiramoto 1968). The reaction may also occur with tyrosine and histidine residues (Peters and Richards 1977). However, various
aldehyde treatments on collagen resulted in hardly any significant difference in the conformation of collagen (Fathima et al 2004).

Oxazolidine having tanning properties is of significant interest to tanning industry. Hydrogen located on $\alpha$-carbon atom of an alkyl group in the 2- position of oxazolidine is active and readily replaced by other groups. The nitrogen of the oxazolidine is basic and forms salts with acids and forms quaternary compounds with alkyl halides. Oxazolidine has tendency to interact with basic amino acids and hydroxyl amino groups. Tetrakis hydroxymethyl phosphonium interacts with amino groups of collagen and to a lesser extent with hydroxyl and amido groups. Tanning with tetrakis hydroxymethyl phosphonium is carried out at pH 5.0 (Covington 1997).

The tanning of skin is achieved by the use of a variety of tanning agents, which are capable of having covalent interactions and forming crosslinks (formaldehyde, glutaraldehyde etc.), coordinate covalent bonds (Cr(III), Al(III)) and hydrogen bonds associated with fiber coating (vegetable tannins). In other words, different protein sites may be involved in the formation of crosslinks, depending on the nature of tanning agent and the stability of the skin matrix against degradation by collagenase.

1.3 BIOPOLYMERS FOR TANNING

Polysaccharides, inexpensive natural biopolymers are widely used as raw materials in several industries (Mehltretter 1967, Laleg and Pikulik 1993, Doublier 1987). Being natural compounds, most polysaccharides are easily biodegradable (Lichtenthaler 2002, Vipin 2008, Hou 2008). To fulfill the demand for tailored end-use applications, the native biopolymers often need to be modified. Biopolymers such as starch, cellulose and alginates were
chosen to convert them into polymeric dialdehydes and used for tanning, as these biodegradable natural polymers contain high degree of functionality.

1.3.1 Starch

Starch is composed of units of dehydrated glucose that are linked with glycoside bonds. There are three hydroxyls attached to carbons at 2,3,6 position in each dehydrated glucose unit as given in Figure 1.1. Among these, the primary-hydroxyl in C₆ has the strongest reactivity and then there are the secondary hydroxyls in C₂ and C₃. There are many reactive groups available in the starch molecule that can react with different chemical reagents. Periodate oxidation of starch selectively cleaves the C₂-C₃ bond between the two adjacent hydroxyl groups, and the 1,2-diol group in glucose is converted into a dialdehyde (Mehltretter 1967, Veelaert et al 1997).

![Figure 1.1 Structure of Starch](image)

1.3.2 Cellulose

Cellulose is a natural polymer that has widespread applications (Laleg and Pikulik 1993, Mehlttretter 1967). A variety of cellulose derivatives are produced by chemical modification (Veelaert 1997, Lichtenthaler 2002, Vipin 2008). Periodate-oxidised-cellulose is prepared under mild aqueous conditions and is characterized by specific cleavage of the C₂-C₃ bond of the
glucopyranoside ring that produces two aldehyde groups per unit as given in Figure 1.2 (Bobbit 1956, Hou 2008, Tang 2005).

![Chemical structure of cellulose](image1)

**Figure 1.2  Structure of Cellulose**

### 1.3.3 Alginate

Sodium alginate is the only natural polysaccharide, which naturally contains solubilising carboxyl groups in each constituent residue and possesses various abilities for crosslinking. It is a linear copolymer consisting mainly of residues of 1, 4-linked α-L-guluronate and β-D-mannuronate acid monomers. These monomers are often arranged in homopolymeric blocks, separated by regions approximating an alternating sequence of the two acid monomers as given in Figure 1.3.

![Chemical structure of sodium alginate](image2)

**Figure 1.3  Structure of Sodium Alginate**

Along the polymer chain the two residues are arranged in an irregular blockwise pattern (Lichtenthaler 2002). There are three types of...
blocks: homopolymeric sequence of mannnuronate (MM blocks) and
guluronate residues (GG blocks) and a region where the two residues alternate
(MG blocks). The relative proportions of these block types are affected by
several factors such as the botanical source, plant maturity, collection site and
seasonal variations (Swarna et al 2006).

1.4 CONVERSION OF BIOPOLYMERS TO POLYALDEHYDES

Polysaccharides like starch, cellulose and sodium alginate are
modified and used in several industries. These polysaccharides can be
oxidized by reagents like sodium periodate, chromic anhydride and
permanganate.

1.4.1 Selective Oxidation Methods

The biopolymers on oxidation, form dialdehydes within the
polysaccharide molecule. Polyaldehydes are prepared by selective oxidation
of the biopolymers that cleave the C$_2$-C$_3$ bond between the two adjacent
hydroxyl groups and the 1, 2-diol group in glucose is converted into a
dialdehyde.

1.4.1.1 Periodate oxidation

There are exhaustive reviews on periodate oxidation of
carbohydrates in general (Bobbitt 1956, Dyer 1956) and of starch in
particular. The periodate ion reacts with the 2,3-glycol group of starch to
cleave the C-C bond with the formation of two carbonyl groups as seen in
Figure 1.4. The carbonyl groups are capable of forming hemiacetal and/or
various hydrated aldehyde structures either inter-molecular or intra-molecular.
Periodate oxidation of cellulose is given in Figure 1.5. The classification of the various types of carbonyl groups in oxidized cellulose according to their rates of oxidation was developed (Neimo et al 1963). Periodate oxy cellulose was found to contain six different types of carbonyl groups.

1.4.1.2 Chromic anhydride oxidation

The oxidation of starch with chromic anhydride in non aqueous system or in 0.2 M sulfuric acid introduces aldehyde, ketone, and carboxyl groups. Potassium dichromate however, is also reported to yield a product containing 60% dialdehyde groups and only 4-5% of carboxyl groups (Waters 1964). In the presence of acid, glycol groups react with chromate to form a cyclic ester. Like the periodate ester, the dichromate ester decomposes to yield two carbonyl aldehyde groups in the case of starch as seen in Figure 1.6.
1.4.1.3 Permanganate oxidation

Little is known about the effects of permanganate on starch. Carbonyl and carboxyl are formed with an apparent maximum rate at pH 10 (Waters 1964). The most satisfactory mechanism proposed for alkaline permanganate oxidation of alcohol groups is the abstraction of a hydrogen atom from the alcohol anion (Stewart and Benjamin 1964) although hydride transfer from the anion is also an attractive possibility as seen in Figure 1.7.

![Figure 1.7 Oxidation of Starch by Permanganate](image)

1.5 CHEMISTRY AND NATURE OF POLYALDEHYDES

1.5.1 Oxidized Biopolymers – Polyaldehydes

Periodate oxidation of the biopolymers selectively cleaves the C2-C3 bond between the two adjacent hydroxyl groups, and the 1,2-diol group in glucose is converted into dialdehyde. Polyaldehydes like DAS, DAC and DSA are thus open chain biopolymers containing dialdehyde groups. The extent of oxidation can be readily controlled, and a complete range of aldehyde derivatives of polysaccharides are made available as the oxidation level varies between 0 and 100%.

1.5.2 Degree of Oxidation – 33, 66 and 99% Oxidized Biopolymers

The degree of oxidation can be readily controlled in polyaldehydes and a complete range of aldehyde derivatives is made available as the oxidation level varies between 0 and 100%. At 100% oxidation hydroxyl groups of each glucose residue in the polyaldehyde is converted to its
corresponding dialdehyde structure; at 50% oxidation half of the glucose residues of the polyaldehydes are converted. One distinguished feature of DAS and DAC is that it is not soluble in water or common organic solvents, even after complete oxidation; the product becomes water-soluble only after reduction of its aldehyde groups to primary alcohols (Swarna et al 2006) or to carboxylic acid groups by chlorite oxidation (Veelaert et al 1997). This insolubility was ascribed to hemiacetal formation of aldehyde groups with starch’s or cellulose’s remaining hydroxyl (Swarna et al 2006) groups. While water-solubility is advantageous for utilizing the aldehyde reactivity of polyaldehyde, fully oxidized polyaldehyde can be solubilised in water only by heating or autoclaving and hydrolysis with acid, alkali and enzyme. However, DSA has the advantage of higher solubility characteristics due to the presence of solubilising carboxyl groups and aldehyde functionality. Solubility results from depolymerisation of the sodium alginate during oxidation.

1.6 COLLAGEN - RAW MATERIAL FOR LEATHER

The main protein in skin is collagen. Other proteins present in native skin are removed during the early stages of the leather making process together with other non-collagenous components. Removal of these non-collagenous proteins is necessary to produce soft leather. Otherwise, the fibre structure will be cemented together during drying. Removal of glycosaminoglycans and particularly proteoglycans is necessary to allow the fibre structure to split apart; that allows diffusion of tanning and post tanning chemicals to produce the range of organoleptic properties or handle or feel required by the consumer. The fibrous structure of hide (from big animals) or skin (from small animals) is obtained by dissolving it in a solution of sodium sulfide buffered with calcium hydroxide and the structure is opened up by prolonging the treatment at pH 12.5; this is followed by the action of proteolytic enzymes (called bating) at pH 9 to break down albumins and globulins.
1.6.1 Importance of Collagen in Leather

The hierarchy of collagen matrix in skin structure is well defined and illustrated in Figure 1.8. Fibers are made of fibril bundles; the fibrils are the lowest level of structure that is visible in intact collagen and they are characterised by a repeating banding pattern, which can be emphasised by staining with heavy metals.

To date, at least 29 types of collagens were identified, each performing a different function in skin or other animal tissue (Okuyama et al 2004). Type I Collagen is characterised by its glycine content, one glycine at every third residue Gly-X-Y, its uniquely high proline content, often next to
glycine in the sequence Gly-Pro-Y and its unique hydroxyproline content, usually next to proline in the sequence Gly-Pro-Hyp. The presence of proline in the sequence causes the chain to twist, forming a left-handed helix. The presence of glycine at every third residue allows three α-helices to twist together in a right-handed triple helix, with the glycine methylene groups situated in the centre of the structure. The presence of the hydroxyproline provides a powerful stabilizing effect by hydrogen bonding.

Figure 1.9  Structure of Collagen at Different Levels
Type 1 collagen, with the triple helical structure consists of two $\alpha_1$ and one $\alpha_2$ chains, each 1052 residues long and is distinguished by minor differences in amino acid sequences as given in Figure 1.9. The structure of collagen was rigorously reviewed by Kadler and Brodsky (Kadler et al 1996, Bella et al 1995). The pattern of charge distribution in the collagen is repeated every 234 residues, although not necessarily the same amino acid sequence, which is called the D period (Figure 1.10).

![Figure 1.10 D-Periodicity of Collagen](image)

1.6.2 Collagen Fibrilogenesis and its Implication

Collagen Types I, II, III, V and XI self assemble into D-periodic cross-striated fibrils (Chapman et al 1990, Prockop and Kivirikko 1995). The assembly of collagen molecules into fibrils is entropy driven process (Kadler et al 1987). These processes are driven by the loss of solvent molecules from the surface of protein molecules and result in assembles with a circular cross-section, which minimizes the surface area/volume ratio of the final assembly. Collagen fibrils can be reconstituted in vitro by warming and neutralizing purified collagen solutions from the cold. In vitro fibrilogenesis is a much simpler process compared to that occurring in vivo, which comprises of a series of cellular events. Studies based on turbidity changes were exploited to examine the in vitro process of fibril formation. This thermally induced
process is distinguished by three phase. First there is lag period during which, there is no apparent change in the solution turbidity or viscosity. This is followed by the growth phase during which the turbidity increases rapidly and distinct fibrillar structure appears. The fibril stabilization phase involves a minimal turbidity change. Many attempts were made using various techniques to determine the nature of aggregate nuclei formed during the lag phase (Silver et al 1979, Bernego et al 1983, Hulmes 1983, Veis and Payne 1988).


1.6.3 Thermal Stability of Collagen

The thermal stability of the collagen triple helix arises from the interchain hydrogen bonds between the amide group of glycine and the carboxyl group of X amino acid residue of the neighboring chain containing the GLY-X-Y triplet repeats and the restriction of the phi and psi dihedral angles of the pyrrolidine ring structure of the frequent repeat of imino acids present in collagen. In addition, several factors were reported to contribute to the stability of the triple helix viz., solvent water molecule-mediated hydrogen bonds (Bella et al 1995), the propensity of the pyrrolidine ring puckering down (C-endo pucker) in the X and up (C-exo pucker) in the Y position of
Gly-X-Y repeats (Berisio et al 2000, Vitagilano et al 2001, Berisio et al 2002). The transition temperature of the collagen triple helix from various species is correlated to the 4(R)-Hypo content (Burjanadze and Veis 1997, Holmgren et al 1998, Jenkins and Raines 2002). However, it is still not possible to consistently explain all the results.

Thermal stability of collagen can be determined for both fibres and solutions (Gustavson 1956, Von Hippel 1967). The denaturation temperatures for the collagen in fibres and in solution are different. The thermal behavior of collagen and leather was studied using micro-shrinkage tester (Borasky and Nutting 1949), differential thermal analysis (DTA) and differential scanning calorimetry (DSC) (McClain and Wiley 1972, Covington et al 1989, Miles and Bailey 2001). Collagen fibres exhibit high resistance to wet heat and the irreversible transition occurs between temperatures of 60-63 ºC, which results in the shrinkage of the fibres to one third of their original length. The heating of native or tanned collagen fibre in a calorimeter gives rise to a sharp peak at a defined temperature. The position, height, width, area and symmetry of the thermogram peak provide valuable information about the denaturation processes. It is also known that collagen fibre that loses its flexibility can be recovered by rewetting. These changes can be correlated with the sorption isotherm, in which the threshold value can be seen as the point at which the resorption curve follows the same pattern as the desorption one. Reaching the irreversible state, one is faced with the rigid material for which the properties are no longer those of the native state.

Thermal stability, as measured for instance by the shrinkage temperature (Tₚ) for the dried collagen is higher than that for the hydrated (Native) state (Grigera and Mogilnar 1983). Native dry collagen can be heated to temperature higher than 140ºC. However, the existence of collagen and collagen like substances having high thermal stability in anhydrous
environment or having other substitutes with more efficient inductive effect (Holmgren et al. 1998) cannot be used as a proof that water is not essential to maintain the native state. The strength of the collagen fibers has to be related to the intrinsic property of the collagen fibrils. The observed increase in the strength of the tanned collagen fibers is correlated to the new crosslinks resulting due to tanning (Usha and Ramasami 2000).

1.6.4 Stability of Collagen Towards Enzymatic Stability

Collagen is an unusually stable protein. Collagen is resistant to all enzymes except collagenase. Triple helical structure of the collagen renders stability against degradation to several proteinases other than collagenases (Seifter and Harper 1971). The stability of the collagen against collagenolytic activity forms the core for tanning. Therefore, the interaction of the tanned collagen with collagenase gains importance in understanding the molecular level mechanism of tanning.

1.6.5 Degradation of Collagen

Controlled degradation of collagen, (Figure 1.11) in live tissues is essential in biological processes (Harrington 1996). The triple helical conformation makes collagen resistant to hydrolysis by several proteinases (Mandl et al. 1953, Seifter and Harper 1971, Woolley 1984). However, native collagen is susceptible to attack by a specific enzyme, collagenase, the protein is rendered susceptible to hydrolysis by other proteases (Von Hipple 1967). True collagenases are enzymes specific to only collagen. Those acting on other protein substrates are named non-specific collagenases (Demina and Lysenko 1996).
Figure 1.11 Degradation of Collagen by Collagenase

Collagenases are synthesized by prokaryotes and eukaryotes (Nordwig 1971). Collagenases are isolated from tissues of vertebrates, crabs, and fly larvae etc and culture liquid of various microorganisms (Eisen et al 1970,1973). Collagenases are categorized as mammalian and bacterial type. Mammalian collagenases play important roles in wound healing, growth regulation, tissue remodeling and resorption. They assist also in invasion of host tissue by rapid degradation of the collagen matrix (Metzmacher et al 2007). Mammalian collagenases cleave native collagen in a specific and characteristic manner. A single scission across all three α-chains of the triple helical tropocollagen (TC) monomers at a specific, sensitive locus approximately three-quarters from the N-terminus to produce TC^A and TC^B fragments was reported. In type I collagen, cleavage occurs at the Gly-Ile bond of α_1 chain and Gly-Leu bond of α_2 chain (position 775-776) (Miller et al 1976). Bacterial collagenases are able to make multiple scissions within the triple helical domain and ultimately reduce them to a mixture of small, dialyzable peptides of 3-6 amino acid residues. Collagenases hydrolyse the non-polar regions of the collagen molecule at neutral pH by cleaving the X-Gly bond of the sequence –Gly-Pro-X-Gly- Pro-X- where X is any amino acid frequently alanine or Hydroxyproline (Grobelny and Galardy 1985). However, it does not hydrolyse the polar regions, which are retained as large
polypeptides consisting of up to 25 amino acid residues. Recently *clostridium histolyticum* collagenase was classified into two categories viz. Class I and Class II collagenases. The sites of cleavage on the collagen molecule were identified to be G-P-R, G, G-A-R-G and G-F-O, G (French et al 1992). The mode of action of tissue collagenases on collagen was studied extensively (Vettakkorumakankav and Ananthanarayanan 1999). The conformation of the triple helix at or near the cleavage site has distinctive features that may be important for recognition and binding of collagenases.

1.7 CURRENT SCENARIO OF POLYALDEHYDE TANNING

Tanning with aldehydes was known for a very long time and aldehydes such as formaldehyde, glyoxal and glutaraldehyde were used to stabilize skin. Aldehydes are excellent crosslinking agents for the electron microscopy study of proteins, cells, etc. Galactose dialdehyde was used as protein crosslinker and was very reactive at basic pH leading to complex reaction mixtures. Large number (more than 1000) of glucose residues are present in each starch molecule, which on 100% oxidation, results in large number of dialdehyde groups that can be used to stabilize the collagen matrix. Studies on the use of DAS as a tanning material were carried out earlier by Nayudamma et al 1961. Swarna et al 2006 used autoclaved DAS and established that hydrolysis of DAS results in lower molecular weight DAS species that result in enhanced tanning conditions. Nayudamma et al investigated the binding of dialdehyde starch to collagen, modified collagen and to amino acids (Nayudamma et al 1961). DAS was used as a pretanning material for tanning, rapid tanning and reuse of such liquors for sole leathers.
1.8 BIOCOMPATIBLE STUDIES FOR TOXICITY BEHAVIOR OF POLYALDEHYDE

Cytotoxicity is the quality of being toxic to cells. Cytotoxicity can be measured by the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole (MTT) assay. MTT assay is a standard colorimetric assay (an assay which measures changes in color) for measuring cellular proliferation (cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials (Azad et al 2005). Yellow colour MTT is reduced to purple formazan as given in Figure 1.12 in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxide or a solution of the detergent sodium dodecyl sulfate in dilute hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by spectrophotometer. The reduction takes place only when mitochondrial reductase enzymes are active, therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

![Chemistry of MTT Assay](image)

**Figure 1.12 Chemistry of MTT Assay**
1.8.1 Cell Viability

Cell viability can be defined as the number of healthy cells in a sample. Whether the cells are actively dividing or are quiescent is not distinguished. Cell viability assays are often useful when non-dividing cells (such as primary cells) are isolated and maintained in culture to determine optimal culture conditions for cell populations (Wing et al 1990). The most straightforward method for determining viable cell number is a direct counting of the cells in a hemocytometer. Sometimes viable cells are scored based on morphology alone; however, it is more helpful to stain the cells with a dye such as trypan blue. In this case, viability is measured by the ability of cells with uncompromised membrane integrity to exclude the dye. Alternatively, metabolic activity can be assayed as an indication of cell viability. Usually metabolic activity is measured in populations of cells by incubating the cells with a tetrazolium salt (MTT, XTT, WST-1) that is cleaved into a colored formazan product by metabolic activity.

1.8.2 Cell Proliferation

Cell proliferation is the measurement of the number of cells that are dividing in a culture. One way of measuring this parameter is by performing clonogenic assays. In these assays, a defined number of cells are plated onto the appropriate matrix and the numbers of colonies that are formed after a period of growth are enumerated. Drawbacks to this type of technique are that it is tedious and it is not practical for large numbers of samples. In addition, if cells divide only a few times and then become quiescent, colonies may be too small to be counted and the number of dividing cells may be underestimated. Cell proliferation is one of the major concerns in evaluating the non-toxic nature of tanning agent; general growth phase of a fibroblast is shown in Figure 1.13. Alternatively, growth curves cannot be established, which is also
time-consuming and laborious. Another way to analyze cell proliferation is the measurement of DNA synthesis as a marker for proliferation. In these assays, labelled DNA precursors (3H-thymidine or bromodeoxyuridine) are added to cells and their incorporation into DNA is quantified after incubation. The amount of labelled precursor incorporated into DNA is quantified either by measuring the total amount of labelled DNA in population, or by detecting the labelled nuclei. Incorporation of the labelled precursor into DNA is directly proportional to the amount of cell division occurring in the culture. Cell proliferation can also be measured using more indirect parameters. In these techniques, molecules that regulate the cell cycle are measured either by their activity or by quantifying their amounts.

![Figure 1.13 Cell Growth](image)

**Figure 1.13 Cell Growth**

1.9 NEED FOR ADDRESSING THE MECHANISM IN POLYALDEHYDE TANNING

The health and safety hazards associated with formaldehyde have led to its being effectively banned as a tanning agent. Glutaraldehyde has come under scrutiny with regard to health and safety implications, and will be phased out of the tanners' options. The use of DAS, DAC and DSA for tanning seems to have an immense potential for the reason that these tanning
agents are eco-acceptable. Leathers tanned using DAS and DAC were reported (Mehltretter 1966, McGuire and Mehltretter 1971), but the effect induced by autoclaving and hydrolysis of DAS has not been explored so far. Although, many researchers have concentrated on gelation characteristics of various starches and DAS, there is no information available on the molecular weight changes occurring in DAS due to autoclaving. Therefore, in this work, the molecular weight changes that occur to DAS, DAC and DSA while autoclaving were investigated. DSA has not been reported so far and we have developed DSA as a tanning agent and explored the possibility of making leathers. Though DAS and DAC were reported to have tanning potency, the enzymatic stability of DAS tanned leathers is yet to be elucidated. The stability of leather against collagenolytic degradation is a fundamental requirement for tanning and hence it is imperative to study the enzymatic stability of DAS, DAC and DSA tanned leathers. Also, earlier reports have not focused on organoleptic properties and changes in the fibre structure orientation due to the treatment of DAS and DAC. Hence, in the present study an attempt to understand the effect of autoclaving on DAS and establish conditions at which the DAS can be an effective tanning agent in providing good thermal, enzymatic stability, physical and organoleptic properties to the leathers was carried out. The need for better understanding of the structural changes involved in the polyaldehyde tanning process has also been stressed. Binding of DAS by functional sites in collagen may be responsible in fixation of the polyaldehyde and the chemical stability of polyaldehyde interacted collagen compound may well arise from specific interactions, many of which have not yet been understood. An approach to the mechanism of molecular theory for tanning by aldehydes would need a fundamental understanding and molecular insight into the process. On the basis of current level of resolution of knowledge in the area, it is difficult to develop convincing molecular
approaches for tanning. The present work has made an attempt to seek further molecular level understanding of polyaldehyde tanning.

1.10 UNDERSTANDING MOLECULAR INSIGHTS IN POLYALDEHYDE TANNING

Understanding the molecular level mechanism of tanning will aid in designing new tanning molecules or new methodology so as to enable the sustainability of the tanning industry from the clutches of pollution problems. Many unanswered questions in the understanding of polyaldehyde tanning were recognized through a critical review of the current level of knowledge in the area. The present study has aimed at some approaches to understand the collagen-polyaldehyde-collagenase interaction more completely. Dialdehyde starch is known to contain different oligomeric species viz. dimer, trimer, tetramer and other higher oligomers. Some species of the polyaldehyde are preferentially bound by collagen from a mixture of complexes. This leads to enrichment of tanning solutions by polyaldehyde complexes. For gaining molecular insight into polyaldehyde tanning, it is necessary to investigate the reactions of collagen with selected and isolated species. A special attribute of skin arises from the pore connectivity and the pore size distribution varying from 8 Å to 500µ. Mass transfer into the hide/skin during the process of tanning is expected to be influenced by several factors like diffusion, fiber swelling, pore size etc. The availability of polyaldehyde near potential reaction sites at and near the pentafibrillar regions may well be affected by molecular dimensions of the different polyaldehyde species.

While the stabilization of collagen by polyaldehyde was correlated with the nature of covalent linkages, there remains a need for studying the secondary structural changes of collagen after crosslinking with polyaldehyde. A holistic understanding of tanning would require the understanding of
processes occurring at both matrix and molecular levels. A need for a rational understanding of how tanning agents with widely varying chemical reactives are able to stabilize collagen has to be recognized.

Aldehydes may well bring about conformational changes in collagen such that collagenase does not recognize the substrate (Fathima et al 2004). It is also possible that polyaldehyde may bring about structural alterations (secondary and quaternary) in the enzyme as well as collagen. If such alterations occur at or near the active sites on the protein and catalytic domain of collagenase, inhibitory effect of polyaldehydes may result in tanning. Structural changes in collagen on reaction with polyaldehyde need to be discussed at the levels of secondary, tertiary and quaternary structures.

The development of an integrated theory of tanning might be both complex and involve investigations on a number of tanning systems. An approach towards such an objective needs to be initiated. The primary objective of the proposed investigation is to initiate an approach towards a conceptual insight into the molecular basis of polyaldehyde integrated theory of tanning.

1.11 BIODEGRADABILITY OF TANNED COLLAGEN

Leather is usually not biodegradable according to the principal world standards. Modified carbohydrate biopolymers as dialdehydes that are biodegradable and toxologically accepted and used as tanning agents. The biodegradability of these biopolymeric tanned leathers in comparison with conventional chrome, vegetable and aldehyde tanned leathers is focused in the present dissertation.
1.12 DEVELOPMENT OF BIODEGRADABLE LEATHERS

Solid waste management is one of the major concerns of the global leather sector. The leather sector, world-wide generates 18 billion square feet of leathers annually, which in one form or the other have to be disposed off finally. Most of the leathers are made from chrome and vegetable tanning system and they are hard for biodegradation. Hence, the solid leather wastes pose a severe threat to the environment. To attain a secured disposal of this solid waste, it is essential to develop leather, which on disposal after usage could easily be biodegradable. Biodegradation is the process of breaking down of organic materials into simple chemicals by microorganisms. The solid leather waste generated from leather processing units and the unused leather goods are generally disposed off in the dump yard that renders the solid waste management system highly inactive because of the non–biodegradability of the finished leathers. Tanning is the process of conversion of a biodegradable material to a non-biodegradable material, thereby the leather acquires resistance towards chemical, thermal and microbiological degradation. Biodegradable leather in the present context is referred to the development of leather using a suitable tanning system, which is easy for biodegradation under certain environmental conditions.

1.13 SCOPE OF THE PRESENT WORK

The present study, aims at developing perceptive mechanism of polyaldehyde tanning, adopting molecular approaches in studying the collagen-polyaldehyde-collagenase interaction. While, polyaldehyde tanning is yet to be elucidated completely, the inadequacy in gaining insights on this tanning system that have the ability to convert hides and skins into biodegradable leather is addressed.
The scope of the present work is

- To investigate the preparation of polyaldehydes like DAS, DAC and DSA from starch, cellulose and sodium alginate as tanning agents for the stabilization of collagen
- To investigate the collagen – polyaldehyde tanning system using different polyaldehydes DAS, DAC and DSA along with select species of DAS with respect to the effect of speciation of polyaldehyde in the extent of stabilization of collagen against collagenase and hydrothermal heat
- To investigate the binary system of collagen – polyaldehyde using DAS, DAC and DSA and representative isolated DAS species with respect to modifications in the conformation and quaternary structure of the protein.
- To investigate the collagenase – polyaldehyde tanning system employing the DAS, DAC, DSA and select species of DAS isolated from the polyaldehyde and the enzyme from Clostridium histolyticum with respect to conformational alterations and inhibition of enzyme activity.
- To study polyaldehyde crosslinked collagen as a substratum for cell growth and viability.
- To develop biodegradable leathers with required characteristics.
- To determine biodegradability of polyaldehyde tanned leathers in comparison with chrome, vegetable and other aldehyde tanned leathers.