INFLUENCE OF DIVALENT IONS ON GELATIN HYDROGEL AND ITS PHYSICOCHEMCAL CHARACTERIZATION

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BY

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CERTIFICATE

This is to certify that the work in the thesis entitled "**INFLUENCE OF DIVALENT IONS ON GELATIN HYDROGEL AND ITS PHYSICOCHEMICAL CHARACTERIZATION"** submitted by **Ms. SRISHTI GUPTA (213BM2024)**, in partial fulfilment of the requirements for the award of M. Tech (Biotechnology) at the National Institute of Technology Rourkela, is an authentic work performed by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any University/Institute for the award of any Degree or Diploma.

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Abstract

In this study gelatin hydrogels and their physicochemical properties are studied after chelation by EDTA. Gelatin is highly biocompatible, biodegradable, non immunogenic and can be modified at amino acid level. But due to its thermoreversible nature gelatin gels are not stable in nature and their mechanical strength is very low. Gelatin contains many divalent ions such as calcium, iron, and copper during denaturation process. Due to chelation activities of these divalent ions are diminished thereby making an EDTA metal ion complex. This process leads to more number of free carboxylic acid strengthening the interaction between cross linking agent and the gelatin gel network, hence increasing the stability and viscoelasticity of the gelatin hydrogels. Various characterization techniques were performed namely swelling studies, FTIR analysis, contact angle measurement, in vitro degradation, rheological studies and Hemocompatiblity tests. All the characterization techniques depicts that purified gelatin gel network when crosslinked with gluteraldehyde forms a more dense and stable network when compared with unpurified gelatin crosslinked with gluteraldehyde. More stable gelatin networks can be used in wide areas of soft and hard tissue engineering.

Keywords: Gelatin, EDTA, Chelation, Crosslinking, Stability

Chapter 1

Introduction

Traditionally, tissue engineering techniques was depended on harvesting of tissues or organs from various sources categorized as autologous (from own tissue), allogenic (from other human donor) or xenogenic (from other species). However, the use of these grafts has encountered many problems and has several limitations. Hence, to solve these problems, various researchers are trying to develop replacement materials which can replace these grafts.

Recent developments in tissue engineering field often involves the use of three dimensional scaffolds which functions as a template for cellular activities to repair rebuild and regenerates the lost or damaged tissues in our human body. These scaffolds act as three dimensional specimens for initial cell attachment and subsequent tissue formation. While there are many biomaterials which can be selected for tissue engineering, the biomaterial selected as three dimensional scaffolds should have characteristics such as biocompatibility, biodegradability, interconnected porosity, mechanical strength and surface chemistry. Naturally derived polymers which are derived from natural sources have been used as scaffolds for tissue engineering. They have gained widespread attention due to their unique characteristics of biocompatibility, ease of processing and their low cost. Some naturally derived polymers used are cellulose, chitosan, agarose, collagen, alginate, gelatine etc. Single component scaffold of chitosan, alginate, gelatine as well as composite scaffolds of alginate-gelatine, chitosan-cellulose, chitosan-alginate, cellulose-agarose, chitosan agarose has been synthesized and their characteristics have been evaluated(Ko, Sfeir, & Kumta, 2010). It has been studied in various papers that these naturally derived polymers in the form of hydrogels are synthesized and are used in various tissue engineering fields.

1.1 Hydrogels

According to the researchers, hydrogels have been defined as water swollen three dimensional cross linked polymeric network produced by simple reaction of one or more monomer. These hydrogels are smart enough to respond to the fluctuations of environmental stimuli such as pH, temperature, ionic strength, presence of enzymes, electric field etc to swell and shrink accordingly. In the swollen state these hydrogels are soft and rubbery in nature resembling the living tissue with high biocompatibility.

The ability of hydrogels to absorb water is due to hydrophilic functional groups that are attached to the backbone of the polymers and their resistance to dissolution is due to the cross links between the network chains(Ahmed, 2013). Both naturally occurring and synthetic materials comes under the definition of hydrogels.

Most of the hydrogels are polymers of carboxylic acid functional group. These acid functional groups stick off the main chain of the polymer. When they are mixed with water, the hydrogen atom reacts and come off as positive ions which leave negative ions along the polymeric chain (Figure 1).

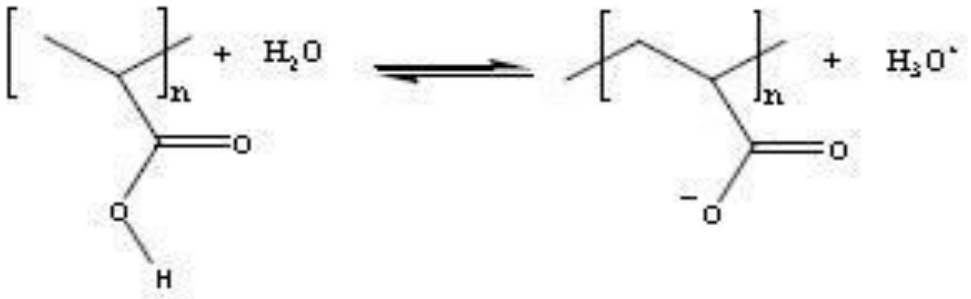


Figure 1: The polymeric chain of hydrogel

These polymeric chains tend to coil up in the solution (Figure 2). The negative charges on the chain repel each other and force the chain to unwind and also attract the water molecules

(Figure 3). The water molecule is covalent, non ionic and has no overall electric charge. However, the oxygen molecule pulls the electron towards itself rendering it slightly negative and hydrogen molecules slightly positively charged. Due to these charges they are attracted by the negatively charged polymeric network. These water molecules stick to the hydrogels and force it to open more.

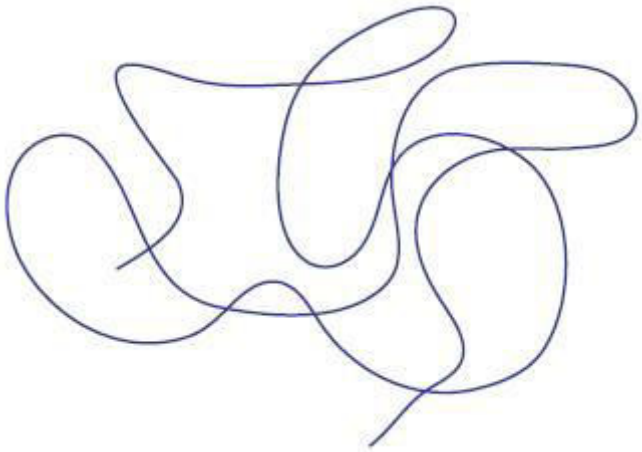


Figure 2: Coiled up hydrogel network

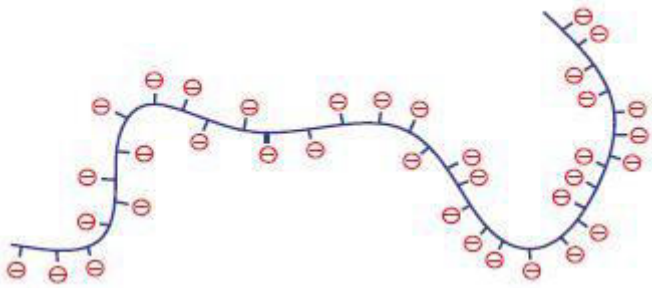


Figure 3: Hydrogel network with negative charge

1.2 Classification of hydrogels

Hydrogels are classified on different basis as described below

1.2.1 Classification based on sources

Based on sources hydrogels are classified in two groups

1) Natural origin
2) Synthetic origin

1.2.2 Classification based on polymeric composition

Based on different method of preparation of hydrogels they are classified as follows:

1) Homopoymeric hydrogels are derived from single type of monomer, which is its basic structural unit(Iizawa et al., 2007).
2) Copolymeric hydrogels are derived from two or more types of monomers with atleast one hydrophilic component. They are arranged in random or alternating configuration along the polymeric network(Philip & Philip, 2010).
3) Multipolymer Interpenetrating polymeric hydrogel (IPN) is composed of two independant cross-linked polymeric component, contained in a polymeric network form. In semi interpenetrating hydrogel network one component is crosslinked while other component is present in non cross linked form(Maolin, Jun, Min, & Hongfei, 2000).

1.2.3 Classification based on different configuration

Based on configuration hydrogels are classified as follows:

1) Amorphous in nature
2) Semicrystalline and
3) Crystalline in nature

1.2.4 Classification based on crosslinking type

Based on the type of crosslinking hydrogels are classified as follows:

1) Physical crosslinking: This type of crosslinking has transient junctions which arises due to polymer chain entanglements or physical interactions such as hydrogen bonds, ionic interactions, or hydrophobic interactions.
2) Chemical crosslinking : this type of crossling has permanent junctions

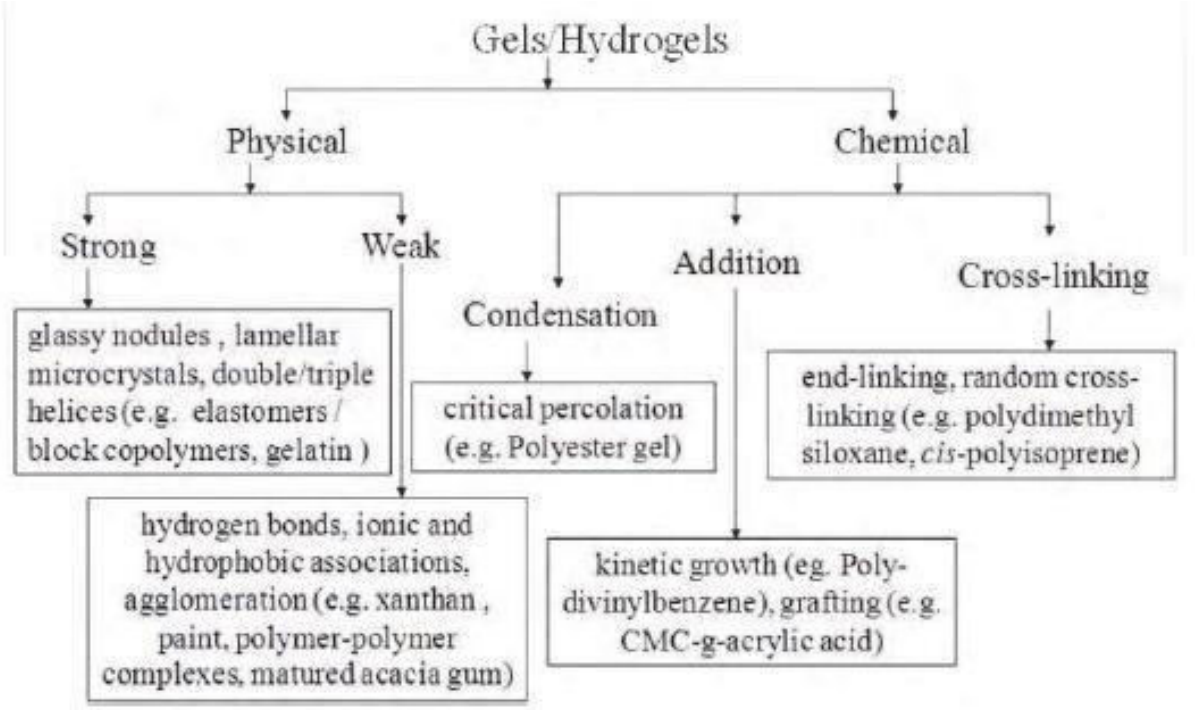


Figure 4: Different types of crosslinking

1.2.5 Classification based on physical appearances

Based on physical appearance hydrogel are classified as follows. This appearances are based on technique of polymerization which are involved during the preparation process:

1) Matrix
2) Film
3) Microspheres

1.2.6 Classification based on network electric charge

Based on presence or absence of electric charges present in crosslinked chain hydrogels are classified as follows:

1) Ionic which includes anionic or cationic
2) Zwitterionic which contains both anionic and cationic groups in structural repeating unit
3) Amphoteric electrolytes which contains both acidic and basic groups
4) Non-ionic

1.3 Preparation of hydrogels

Cross linked networks of either natural or synthetic polymers are prepared by various preparation techniques namely physical crosslinking(Hennink & Van Nostrum, 2012), chemical crosslinking(Barbucci, Leone, & Vecchiullo, 2004), radiation crosslinking(Fei, Wach, Mitomo, Yoshii, & Kume, 2000) and grafting polymerization(Said, Alla, & El-Naggar, 2004).

1.3.1 Physical crosslinking

Due to ease of production and no use of crosslinkers researchers are showing high interest in physical or reversible gels. Careful selection of various parameters such as hydrocolloid type, pH can lead to wide range of gel textures. Different methods of physically crosslinking a hydrogels are as follows

1.3.1.1 Cooling/heating a polymeric solution

When cooling a hot solution of gelatine or carrageenan mixture physically crosslinked gels are formed. These gels are formed due to helix formation and formation of junction zones(Funami et al., 2007). Carrageenan or gelatine solutionsare present as random coils in hot solutions which are above the melting transition temperature. Upon cooling they

transforms to helical structures (Figure 5). Hydrogels are also prepared by warming the polymer solutions which causes block polymerization. One of the example is glycol polylactic acid glycol(Hennink & Van Nostrum, 2012).

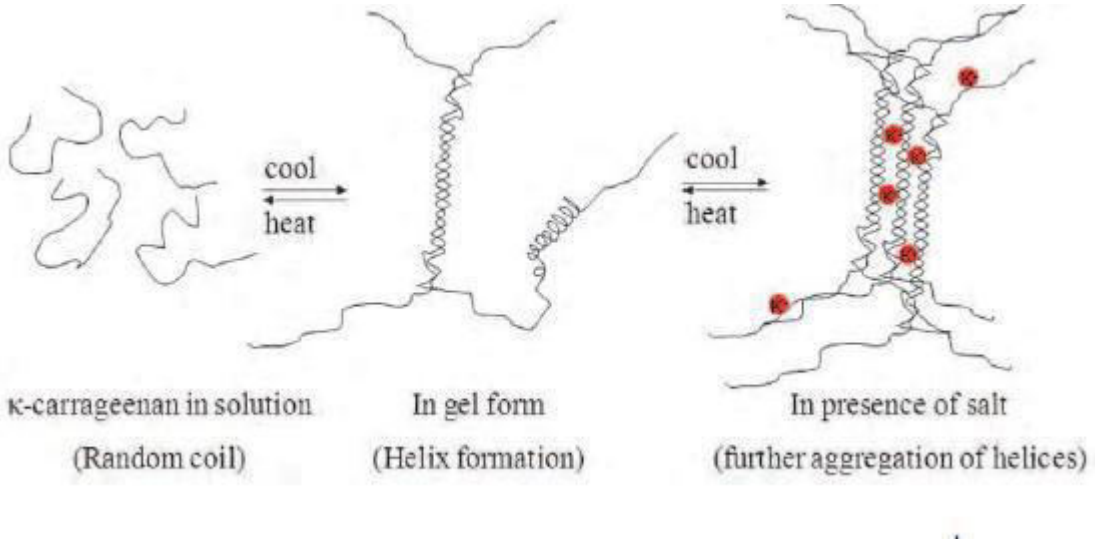


Figure 5: Physical crosslinking of carrageenan solution

1.3.1.2 Ionic interactions

Crosslinking of ionic polymers occurs by addition of divalent or trivalent counter ions. This method underlies the principle of ionotropic gelation where polyelectrolyte solution like sodium alginate is gelled with multivalent ions of opposite charges such as calcium (Figure 6). Some of the other examples are chitson dextran hydrogels(Hennink & Van Nostrum, 2012), chitosan polylysine(Bajpai, Shukla, Bhanu, & Kankane, 2008).

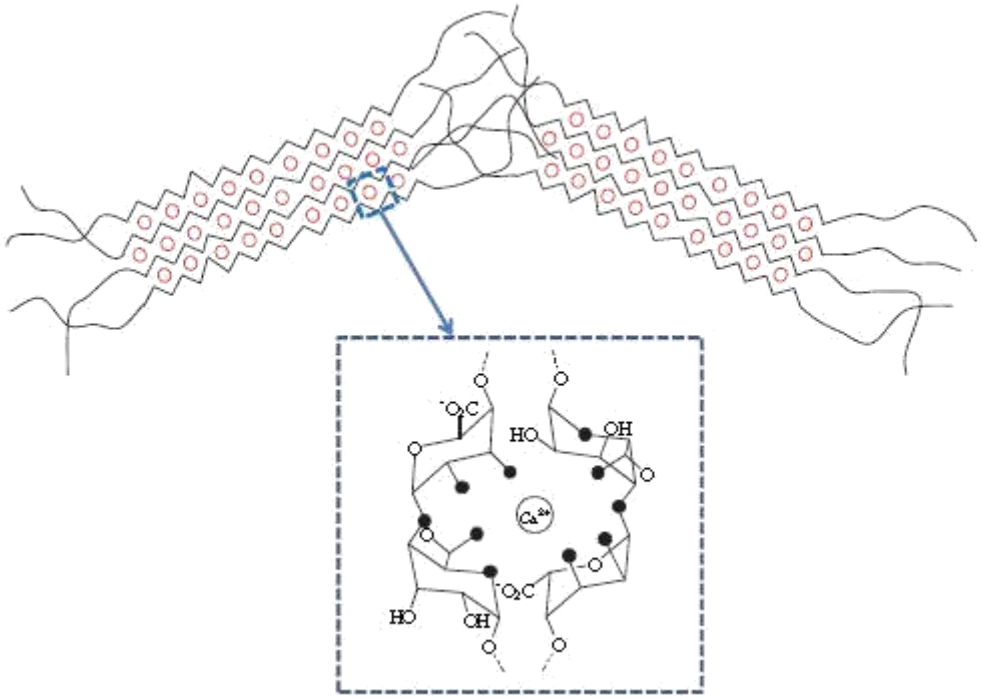


Figure 6: Ionotropic gelation

1.3.1.3 Complex Coacervation

When polyanions and polycations are mixed complex coacervate gels are formed (Figure 7). Principle behind this method is polymers with opposite charges stick together and depending on concentration and pH, forms soluble or insoluble complexes. One of the examples is coacervating polyanionic xanthon with polycationic chitosan(Esteban & Severian, 2000).

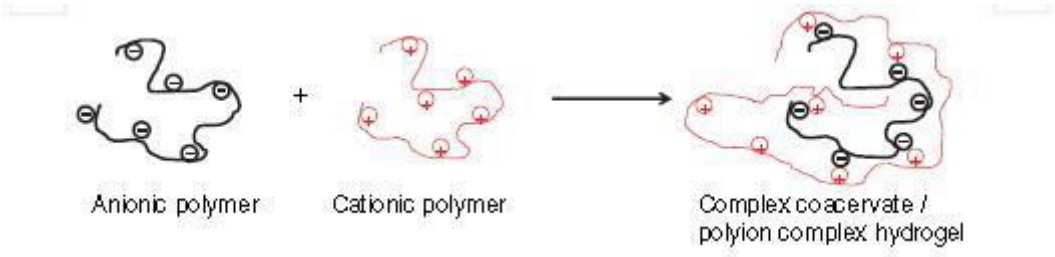


Figure 7: complex coacervartion

1.3.1.4 Hydrogen bonding

Hydrogen bonded hydrogels are prepared by lowering the pH of the carboxylic containing polymeric aqueous solution. Some of the examples are carboxymethyl cellulose dispensed in hydrochloric acid(Takigami et al., 2007),carboxymethylated chitosan, polyacrylic acid and polyethylene oxide (PEO-PAAc)(Hoffman, 2012). The principle involved in carboxymethyl cellulose gel formation is replacement of sodium in carboxymethylcellulose with hydrogen in the acid to form hydrogen bonds (Figure 8). These bonds reduces the solubility of carboxymethylcellulose in water and leads to formation of an elastic hydrogels.

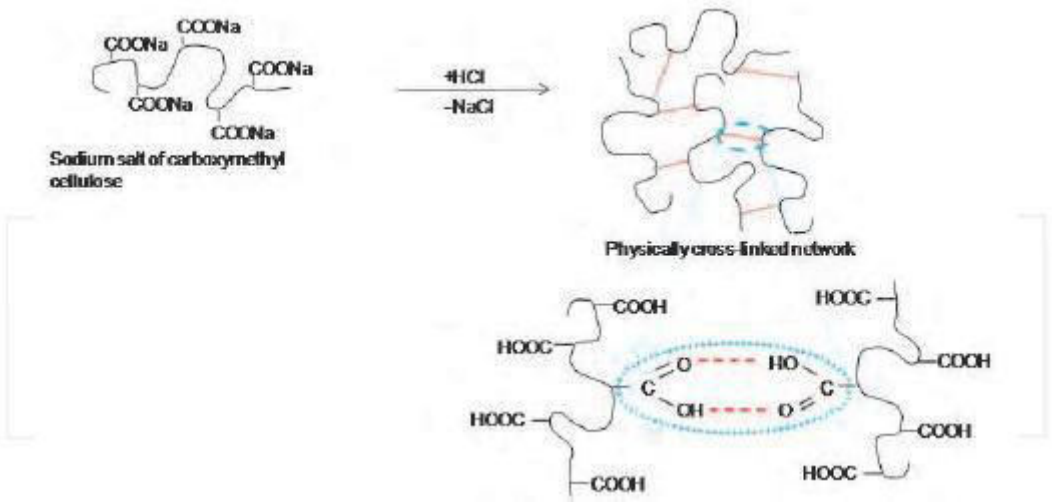


Figure 8: Hydrogels formed by hydrogen bonding

1.3.1.5 Heat induced aggregation

This method predominantly applies to Arabic gums which have some 2-3% proteins in their internal structures(Williams & Phillips, 2006). Due to heat treatment aggregation of proteinaceous components occurs which results in formation of hydrogels with high molecular weight, high water absorbing capacity and enhanced mechanical properties(Al-Assaf, Phillips, Aoki, & Sasaki, 2007). Hydrogels can be produced with precise structural dimensions by controlling the agglomeration of the proteinaceous components in the solution.

1.3.1.6 Freeze thaw

Hydeogels can also be physically crosslinked by freeze thaw cycles.the principle involves formation of microcrystals in the hydrogel structure. Some of the examples are polyvinyl alcohol and xanthan(Giannouli & Morris, 2003).

1.3.2 Chemical crosslinking

Chemical crosslinking of hydrogels involves the reaction of their functional groups such as OH, COOH, NH_2 with various types of crosslinking agents such as gluteraldehyde, dihydrazide etc. This type of crosslinking also involves grafting of monomers on the back of polymers(Gulrez, Phillips, & Al-Assaf, 2011). Use of crosslinking agent to link polymer chains is also one the method invoved. Major chemical methods to prepare hydrogels are stated below.

1.3.2.1 Chemical crosslinkers

Crosslinked polymers of various natural or synthetic polymers are prepared by using various crosslinkers such as gluteraldehyde, carboimides etc. The principle that underlies the formation of crosslinked hydrogels involves the introduction of new molecules between the polymeric chains (Figure 9). One of the examples is carrageenan and acrylic acid can be crosslinked by using 2-acrylamido-2-methylpropanesulfonic acid resulting in formation of hydrogels which are used for targeted drug delivery(Pourjavadi & Zohuriaan-Mehr, 2002).

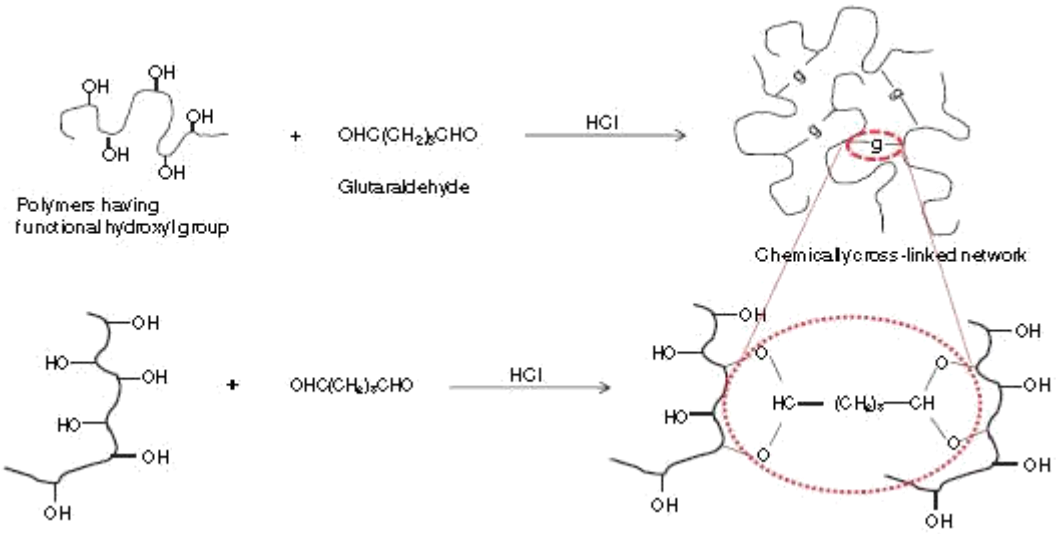


Figure 9: Hydrogels formed by using crosslinkers such as gluteraldehyde

1.3.2.2 Grafting

This process involves polymerization of the monomer on the backbone of the polymer. These polymeric chains are activated by several methods such as chemical treatments or high energy radiations. Branching and crosslinking occurs due to the growth of functional monomers on the activated polymers (Figure 10). Grafting is mainly of two types that is chemical grafting and radiation grafting.

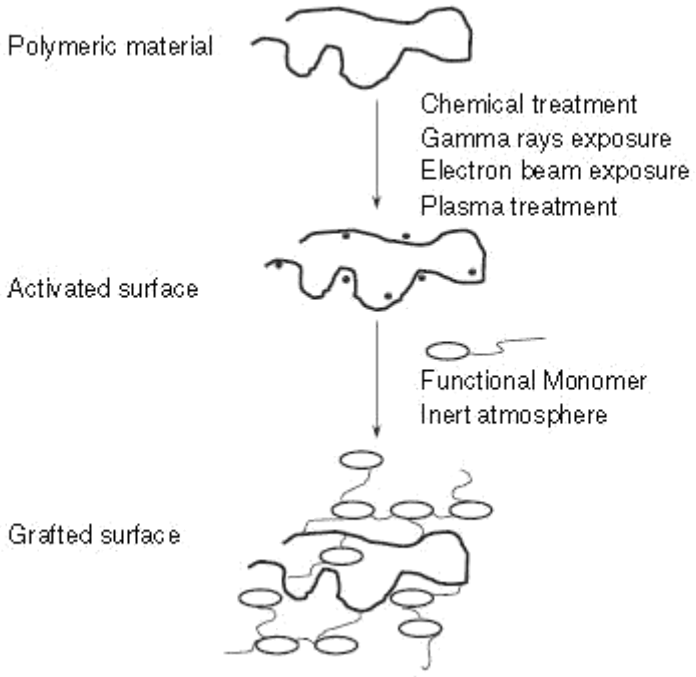


Figure 10: Grafting method for preparation of hydrogels

In radiation grafting polymeric backbone is activated by a chemical reagent. One of the examples is starch grafted with acrylic acid by using N-vinyl-2-pyrrolidone which are used as drug and vitamin delivery systems in the small intestine(Spinelli et al., 2008).

In radiation grafting polymeric backbone is activated by high energy radiations such as gamma radiation and electron beam. One of the examples is preparation of caboxymethylcellulose hydrogel by grafting with acrylic acid in the presence of electron beam(Said et al., 2004). This electron beam irradiation initiated the free radical polymerization of acrylic acid on carboxymethylcellulose backbone.

1.3.3 Radiation crosslinking

This technique is mainly based on production of free radicals in the polymers followed by the exposure to the high energy source such as x ray, gamma ray or electron beam. This technique is widely used as it does not involve the use of chemicals, and sterilization and modification can be attained in single step making it cost effective. This process helps in

modification of biopolymers which are used in number of biomedical applications(Lugao & Malmonge, 2001).

1.4 Application of hydrogels

Hydrogels are mainly used in tissue engineering, pharmaceuticals and biomedical field. These hydrogels have been used in various applications such as drug delivery, agriculture, sanitary pads, wound dressing, ophthalmic applications, dental implants, and injectable polymeric systems (Benamer, Mahlous, Boukrif, Mansouri, & Youcef, 2006; Nho & Lee, 2005; Rosiak & Yoshii, 1999). Various applications involves use of various biomaterials of natural or synthetic origin (Gulrez et al., 2011). Some of the examples are wound care involves use of polyurethane, polyethylene glycol, polypropylene glycol (Rosiak & Yoshii, 1999), drug delivery system involves use of starch, poly(vinylpyrroliodone), polyvinyl alcohol, acrylic acid, carboxymethylcellulose (Benamer et al., 2006; El-Naggar, Alla, & Said, 2006; Kumar, Sasmal, & Pal, 2008; Nho & Lee, 2005; Said et al., 2004), dental implants involves the use of hydrocolloids, and tissue engineering implants involves the use of polyvinyl alcohol, hyaluronan, and collagen(S. J. Kim, Hahn, Kim, Kim, & Lee, 2005; Shu, Liu, Palumbo, Luo, & Prestwich, 2004).

Chapter 2

Literature review

2.1 Gelatin

Gelatin is a protein which is obtained by partial hydrolysis and denaturation of collagen extracted from the boiled bones, organs, connective tissues and some intestines of animals such as domesticated cattle, horses and pigs. The natural bonds present between the collagen strands are broken in this process to a form which can be rearranged easily. Hence triple helical structure of collagen is broken down to random gelatin coils.

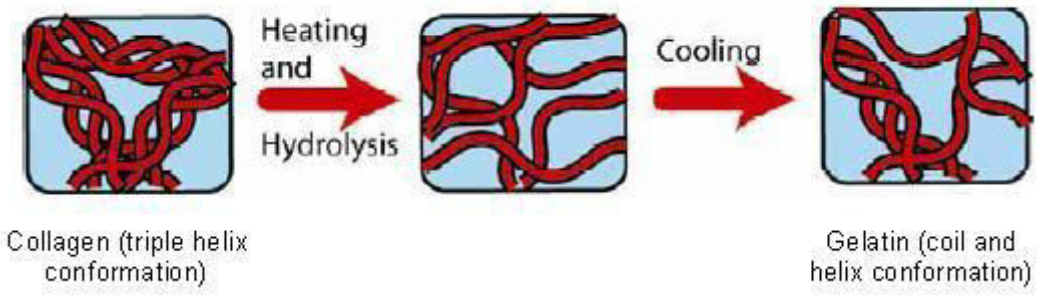


Figure 11: Denaturation process of collagen to obtain gelatin

Gelatin is composed of 50.5% carbon, 6.8% hydrogen, 17% nitrogen and 25.2% oxygen(Handbook). The chemical composition of collagen is maintained after the formation of gelatin despite the loss of macromolecular organization. It is a polypeptide having $-(Gly - X - Pro)_n$ where X represents the amino acids, mostly lysine, methionine, arginine and valine. The polypeptide chain of gelatin constitutes of one third of glycine residue and another one third as either proline or hydroxyproline.

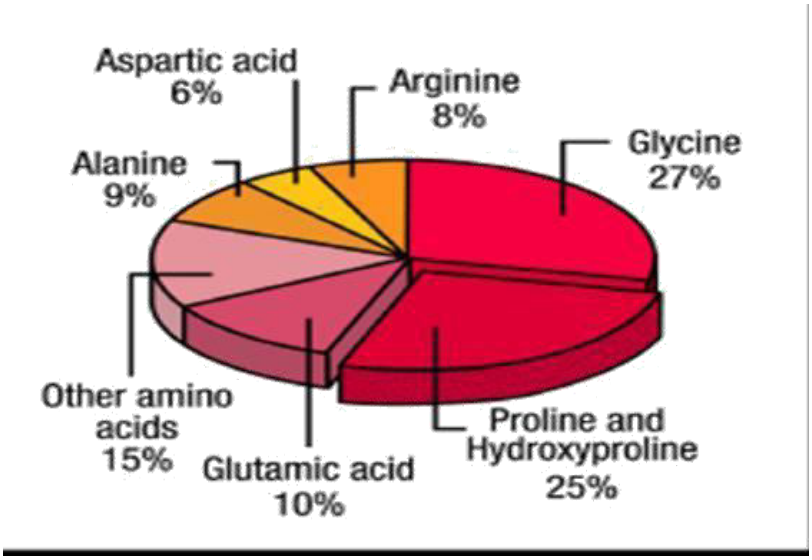


Figure 12: Amino acid composition of Gelatin

	Type A (Porkskin)		Type B (Calf Skin)		Type B (Bone)	
Alanine	8.6	10.7	9.3	11.0	10.1	14.2
Arginine	8.3	9.1	8.55	8.8	5.0	9.0
Aspartic Acid	6.2	6.7	6.6	6.9	4.6	6.7
Cystine	0.1		Trace		Trace	
Glutamic Acid	11.3	11.7	11.1	11.4	8.5	11.6
Glycine	26.4	30.5	26.9	27.5	24.5	28.8
Histidine	0.9	1.0	0.74	0.8	0.4	0.7
Hydroxylysine	1.0		0.91	1.2	0.7	0.9
Hydroxyproline	13.5		14.0	14.5	11.9	13.4
Isoleucine	1.4		1.7	1.8	1.3	1.5
Leucine	3.1	3.3	3.1	3.4	2.8	3.5
Lysine	4.1	5.2	4.5	4.6	2.1	4.4
Methionine	0.8	0.9	0.8	0.9	0.0	0.6
Phenylalanine	2.1	2.6	2.2	2.5	1.3	2.5
Proline	16.2	18.0	14.8	16.4	13.5	15.5
Serine	2.9	4.1	3.2	4.2	3.4	3.8
Threonine	2.2		2.2		2.0	2.4
Tyrosine	0.4	0.9	0.2	1.0	0.0	0.2
Valine	2.5	2.8	2.6	3.4	2.4	3.0

Figure 13: Amino acid composition of Gelatin

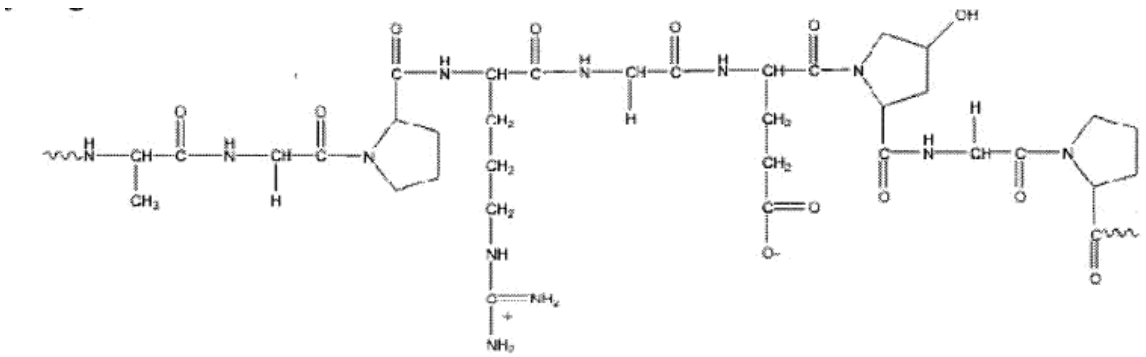


Figure 14: Gelatin Structural Unit

Gelatin when mixed with water forms a semisolid colloidal gel. Gelatin is thermo reversible in nature, hence when heated it remains in liquid state whereas when cooled it becomes solid in nature. Gelatin solution shows streaming birefringence and viscoelastic flow. These gelatin gels exist over a very small range of temperature the upper limit being the melting point of the gel, that depends on gelatin grade and concentration and the lower limit being the freezing point at which ice crystallizes. Gelatin is also found to be soluble in aqueous solution of polyhydric alcohols such as propylene glycol and glycerol. It is soluble in highly polar organic solvents such as formamide and acetic acid. Gelatin is found insoluble in less polar solvents such as acetone, benzene, and primary alcohols(Chanchal et al., 2014)

Gelatin is amphoteric in nature. In acidic solutions gelatin is positively charged whereas in alkaline solutions these are negatively charged(Kenchington & Ward, 1954). The pH where net charge is zero is known as isoelectric point (IEP). Type A gelatin has isoelectric point between pH 7 to pH 9 whereas type B gelatin has isoelectric point between pH 4.7 and pH 5.4(Chanchal et al., 2014; Keenan, 1994)

2.2 Cross linking of Gelatin hydrogels

The control of network formation of gelatin plays a very important role in optimizing specific characteristic of gelatin gels. Chemical crosslinking occurs with the help of various cross

linking agents which are able to bind with the functional group of gelatin side chain to improve mechanical properties, thermal resistance and to decrease its solubility in water. Earlier crosslinking agents such as formaldehyde was used which was toxic in nature, hence less toxic agents such as gluteraldehyde, carbodiimide and genipin are used to avoid the cytotoxicity problems. Gelatin hydrogels are also cross linked by heat treatment or irradiation with ultraviolet light(Zandi, 2008).

Gelatin hydrogels are usually cross linked with crosslinking agents such as gluteraldehyde to improve its elasticity, stability and its consistency. These cross linking agents cause toxic effect on physiological environment where the hydrogels are implanted. Hence there is a need to develop a biocompatible mild cross linked hydrogel(Koh, Yong, Chan, & Ramakrishna, 2008). In one of the paper, a new approach was introduced, where interpenetrating network of gelatin and PVA (Polyvinyl alcohol) hydrogel was prepared by combination of enzymatic crosslinking using transglutaminase enzyme and physical freeze thawing method and results showed god physical and mechanical strength and excellent biocompatibility and proliferation of fibroblast cell cultures(Hago & Li, 2013).

Cross linking of gelatin hydrogels increases the stability of the hydrogels and reduces the dissolution of the gelatin(Welz & Ofner, 1992; Yannas & Tobolsky, 1967). This crosslinking also leads to formation of a dense structure with high molecular weight(Mwangi & Ofner III, 1995). In one of the studies, various effects of pH, temperature, crosslinking and backbone modification of gelatin hydrogel is studied, where gelatin is cross linked with different concentration of gluteraldehyde and the hydrogel is concluded to be appropriate for cell/drug carrier matrices(Einerson, Stevens, & Kao, 2003).

One of the limitations of gelatin hydrogels in the field of tissue engineering is its solubility in aqueous media. Hence to overcome this limitation cross linking of gelatin hydrogels is

done(Di Silvio, Gurav, Kayser, Braden, & Downes, 1994). One of the studies demonstrates preparation and characterization of pectin/gelatin membranes cross linked with gluteraldehyde for wound dressing. Physicochemical characterization of these membranes demonstrated its use in the wound care and these membranes were found to have cytocompatiblity with B16 melanoma cells(Mishra, Majeed, & Banthia, 2011).

Gelatin hydrogels have gained high interest in tissue engineering field due to its high biocompatibility, biodegradability and non immunogenic properties(Van Den Bulcke et al., 2000). Various composition based on gelatin are prepared and are used in vast biomedical areas such as drug delivery, scaffolds for tissue engineering, wound dressing as well other applications such as in fields related to food(Pal, Banthia, & Majumdar, 2007). One such composition studied was interpenetrating polymer network based on gelatin and alginate and cross linked with polyacrylamide which act as superabsorbent materials for soft tissue engineering(SERAFIM et al.). Another study synthesized a biocomponent hydrogel using methacylyamide modified gelatin and polyacrylamide which was cross linked using photoinitiation(SERAFIM et al.).

Due to hydrophilic nature of gelatin, it allows simple and rapid cell seeding, and these seeded cells maintain viability and their function in the culture. Hence gelatin hydrogels have been used widely to culture adherent cells(Wang et al., 2006). Although gelatin has high cytocompatiblity its applications on culturing of cells are limited due to its brittleness(Chang, Raghavan, & Hussain, 1998). To overcome this, in one of the study hydrogel was prepared by mixing mixture of gelatin and PVA (Poly vinyl alcohol) using gamma ray irradiation technique and it was found that fibroblast cultured in these hydrogels showed improved growth ratios and spreading ability(You et al., 2007).

Recently chitosan gelatin blend is cross linked with gluteraldehyde to form scaffolds for hepatic tissue engineering. This scaffold is prepared by rapid prototyping, micro replication and freeze drying techniques forming a three dimensional porous scaffold having well organized fluidic channels and hepatic chambers(Jiankang et al., 2009)

Gelatin hydrogels have been used for wound dressing and wound repair as gelatin hydrogels have shown rapid epithelialisation and growth factors required can be easily incorporated without any loss of the activity of gelatin hydrogel(Schacht, 2004).

4.3 Gluteraldehyde

Gluteraldehyde also known as gluteral, 1,5-pentanedione, potentiated acid gluteraldehyde, sonacide, and gluteraldehyde is an organic compound with formula $CH_2(CH_2CHO)_2$. It is a pungent, colourless oily liquid which has wide range of application such as sterilization of medical equipment, antimicrobial activity in waste water treatment, preservatives in cosmetics, in preparation of grafts, cross linking agent, and as a tissue fixative in histology. It is mostly available in aqueous solutions where the aldehyde groups are hydrated.

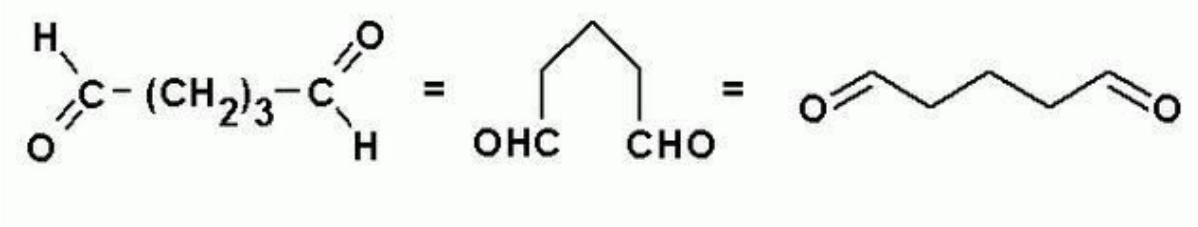


Figure 15: Three representation of monomeric gluteraldehyde

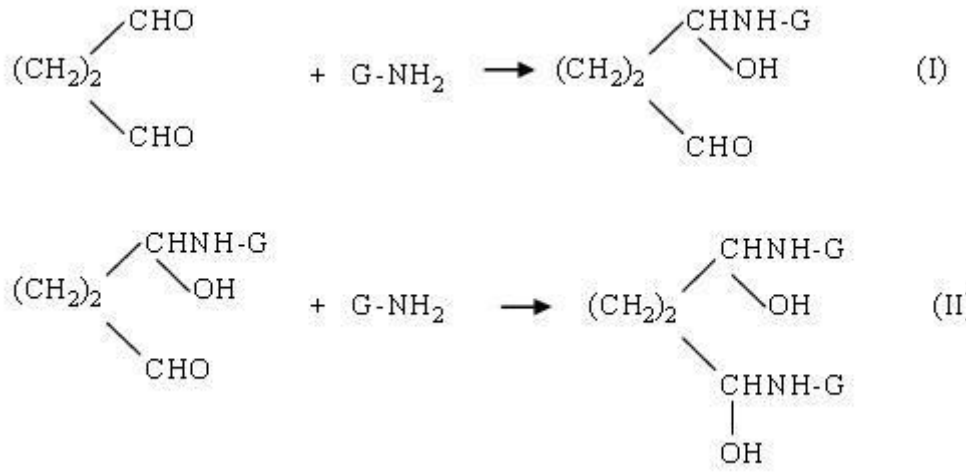
Gluteraldehyde is so far most widely used crosslinking agent for gelatin hydrogels due to its excellent efficiency on stabilization of collagenous material which enables the hydrogels to

achieve high strength(Khor, 1997) and water resistance(Liu, Liu, Liu, Fishman, & Hicks, 2007) and its low cost(Farris, Song, & Huang, 2009). It also reduces the cytoxicity of the material when used in low concentration(Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001).

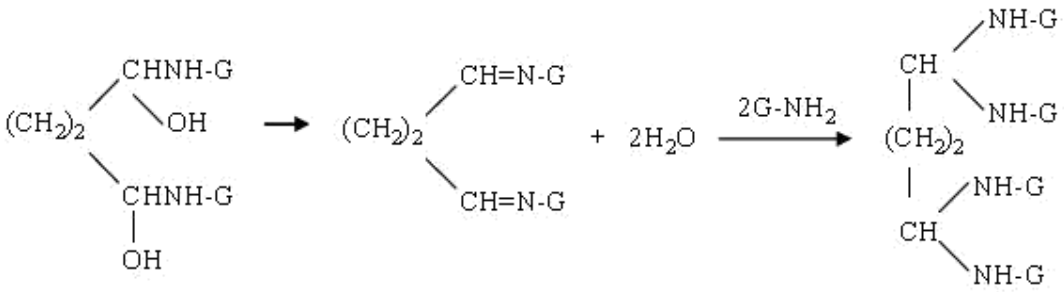
It has been widely accepted that cross linking of gluteraldehyde with gelatin is mediated through the unprotonated ε-amino groups of lysine and hydroxylysine and the amino groups of the N-terminal amino acid(Schrieber & Gareis, 2007). Hence the pH value of the medium is a major factor to control the cross linking reaction. At high pH value, few amino groups are protonated, hence more number of amino groups are free for cross linking reaction whereas at low ph value more number of protnated amino groups are present therby decreasing the crosslinking capacity of gluteraldehyde solution(Farris, Schaich, Liu, Piergiovanni, & Yam, 2009).

Gluteraldehyde as a crosslinking agent is a fast hardener and has two possibilities to cross link with gelatin hydrogel(Zandi, 2008). They are as follows:

1) With the aldehyde group



2) Gluteraldehyde half link could react with other amino group through a Schiff base reaction.



2.4 Gelatin and Its Mechanical Properties

Gelatin is highly biocompatible, biodegradable, non immunogenic, and has great capacity of modification at amino acid level. Due to thermoreversible nature of gelatin gels, the non covalent interactions are easily broken at temperature higher than 30-35°C and the gel network is destroyed. Hence gelatin hydrogels have low shape stability, low elasticity and poor mechanical strength which limit their applications at physiological temperature of 37° C.

To increase the stability and mechanical property of gelatin hydrogels various methods are used. Gelatin gels are covalently cross linked by using cross linking agents such as gluteraldehyde, carbodiimide that couples the carboxyl group with amino group forming stable amide bonds. But this type of cross linking can generate domains with non uniform cross linking(Finch & Jobling, 1977). In one of the studies effect of cross linking agent gluteraldehyde was studied on swelling behaviour and mechanical properties of gelatin hydrogels. It was shown that due to increase in the crosslinking agent concentration swelling decreases. Increase in the concentration of gluteraldehyde also affected the mechanical properties, leading to decrease in toughness and elasticity and increase in the stiffness of gelatin hydrogels (Lou & Chirila, 1999).

Another method was to introduce the polymer along with gelatin gel to overcome the restrictions in the application of gelatin gels. In one of the studies cellulose nanowhiskers

were used to cross link gelatin matrix which led to increase in its mechanical properties and resistance to thermal degradation(Dash, Foston, & Ragauskas, 2013). In another study poly lactic co-glycolic acid microspheres were introduced into gelatin hydrogels to improve its tensile strength(Finch & Jobling, 1977). However, these methods might produce undesirable side effects such as decreasing the non immunogenic property of gelatin hydrogels.

Some researchers studied that the mechanical properties and stiffness of gelatin films are related with the triple helix structure regeneration, which can be detected by differential scanning calorimetry, polarimetry and X-ray. Hence it was concluded that if the process of gelatin renaturation can be controlled its mechanical properties can also be controlled. The mechanical property of gelatin films is also influence by the variation in the pH. It was concluded that at pH greater than 9 and less than 5 Young modulus of the gelatin films decreased drastically whereas when cross linked with genipin young modulus increases significantly (Peptu, Buhus, Popa, Perichaud, & Costin, 2010)

Another study showed that the stability and mechanical strength of the gelatin hydrogel can be improved by removing its divalent metal ions using Chelex Resin. Gelatin hydrogels have many divalent metal ions such as copper, calcium, zinc and iron that form ionic bond with the carboxylic group present in the gelatin polypeptide chain thereby affecting the organization of the gelatin gel network. In this study it is shown that if the divalent ions are removed from the gelatin gel, carboxylic group will be free and they can freely react with amine groups within and between polypeptide molecules thereby making the electrostatic interactions strong in nature between them. Removing the divalent ions also improves the cross linking density, when cross linked chemically(Xing et al., 2014).

2.5 EDTA (Ethylenediaminetetraacetic acid)

Chelation is a process where a particular molecule or ion binds with metal ions. Sequestering agents also known as chelators, chelants, chelating agents are a specific type of organic complexing agents which forms two or more coordinate bonds with a single metal atom and sequesters multiple polyvalent cations. During bond formation these chelating agents act as polydentate ligands.

Various application of chelation are they are used in chelation therapy for removing toxic metals from human body, used as a nutritional supplement, used in fertilizers, used in detergents and used as contast agent in MRI scanning.

One of the chelators used is ethylenediaminetetraacetic acid (EDTA). It is an aminopolycarboxylic acid which is water soluble and colurless in nature. It acts as hexadentate ligand which has the ability to sequester metal ions such as iron and calcium. EDTA ie present in two forms namely disodium EDTA and calcium disodium EDTA. When metal ions bind to this hexadentate ligand it diminishes the activity of the metal ions present in the solution.

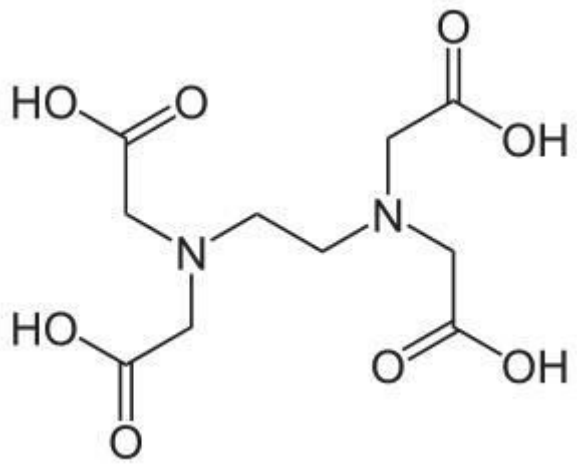


Figure 16: 2D structure of EDTA

In acidic pHs EDTA is present in fully protonated form whereas at higher pHs (acidic medium) EDTA is present in deprotonated form

Mechanism of Chelation

EDTA is present in its protonated form $EDTA^{4-}$ during chelation process. It is a member of aminopolycarboxylic acid family of the ligand. It binds to the metal ion through its four amine and two carboxylate group present in the protonated form, hence forming an octahedral geometry.

Figure 17: EDTA metal ion complex

In tissue engineering field EDTA is also use as crosslinking agent for the formaton of various type of scaffolds. In one of the studies hydrrogels were prepared and characterized using cellulose acetate which were cross linked with EDTA dianhydride and catalysed with triethylamine(Senna, Novack, & Botaro, 2014).

Another study has prepared hydrogels by polymers of acrylic acid, 2-Acrylamidoglycolic Acid, and 2-Acrylamide-2-Methyl-1-Propanesulfonic Acid by cross linking them with EDTA. The cross linking affected the rate of intramolecular propogation(B. Kim, Hong, & Chang, 2014).

Some researchers have prepared chitosan EDTA films improving the physicochemical properties of the chitosan films. EDTA having OH and COOH groups interacts with NH_3 group of chitosan making it stronger in nature. The swelling of the films were decreased and mechanical studies showed high flexibility and recovery after deformation is found to be high(Jones, 2009; Singh, Suri, Tiwary, & Rana, 2012).

Chapter 3

Materials and Methodology

3.1 Materials Used

Gelatin was purchased from HiMedia. Disodium Ethylenediaminetetaacetic (EDTA $Na_2.H_2O$) was purchased from HiMedia. 25% aqueous solution of gluteraldehyde was purchasec from Lobie Chemicals.

3.2. Methodology

3.2.1 Preparation of gelatine hydrogels

3.2.1.1 Preparation of unpurified gelatin gel

Unpurified gelatin was prepared by mixing 20%w/v gelatin i.e 20g of gelatin in 100ml of water with 30ul of gluteraldehyde that act as crosslinking agent during the preparation of gelatin hydrogels. Briefly, 20%w/v solution of gelatin was first mixed thoroughly in magnetic stirrer at 300 rpm at 60°C for 30 minutes. After the solution is mixed 30ul of gluteraldehyde is added to the solution and the solution is casted in a petri plate and the gelation time was noted.

3.2.1.2 Preparation of purified gelatin gel without crosslinking agent

3.2.1.2.1 Preparation of 0.5M EDTA solution

186.12g of EDTA.Na_2.2H_2O was dissolved in ionized water to make up to the volume of 1000ml. The pH of the solution was set to 8 while mixing 20g NaOH and stirring the solution vigorously. This solution was then autoclaved for 20 minutes. The pH of the solution was adjusted to 8 as at this pH only EDTA is dissolved completely in the solution.

3.2.1.2.2 Preparation of gelatin hydrogel

Purified gelatin gel was prepared by mixing 20%w/v gelatin solution with different concentration of (0.2M, 0.3M, 0.4M, 0.5M)EDTA solutions. Briefly, 20%w/v gelatin solution was mixed with 0.2M, 0.3M, 0.4M, 0.5M EDTA concentration in a magnetic stirrer at 300 rpm at 60°C for 2 hours. Once the solution is mixed thoroughly it is casted in petri dishes and kept for gelation for 12 hours at room temperature.

S.no	Gelatin Concentration	EDTA concentration	Gel Formation
1.	20%	0.2M	Loose gel structure is formed
2.	20%	0.3M	Loose gel structure is formed
3.	20%	0.4M	Firm gel structure formed
4.	20%	0.5M	Firm gel structure formed

Table 1: Purified gelatin hydrogel samples

3.2.1.3 Preparation of purified gelatin gel with crosslinking agent

Purified gelatin gels which are prepared by addition of different concentration EDTA were crosslinked using different concentrations of 25% aqueous gluteraldehyde solution. Briefly,

20% w/v gelatin solution was mixed with different concentrations of EDTA in magnetic stirrer at 300rpm 60°C for 2 hours and after this different concentration i.e. 10ul, 20ul, 30ul, 40ul and 50ul of 25% aqueous gluteraldehyde solution was added to the solution and casted in a petridish for gelation for 12 hours at room temperature.

S.no	Gelatin Concentration	EDTA concentration	Gluteraldehyde concentration	Gel Formation
1.	20%	0.5M	10µl	Gel Formed
2.	20%	0.5M	20µl	Gel Formed
3	20%	0.5M	30µl	Gel Formed
4	20%	0.5M	40µl	Rapid Gel formation (aggregates)
5.	20%	0.5M	50µl	Rapid Gel formation (aggregates)

Table 2: Purified gelatin gel samples with gluteraldehyde cross linking

Hence final concentration of gluteraldehyde used for crosslinking was considered to be 30µl and different EDTA concentration of 0.2M, 0.3M, 0.4M and 0.5M was used for preparation of purified gelatin hydrogels.

3.2.2 Characterization Studies

3.2.2.1 Measurement of Ion Content

Ion content present in the hydrogel samples both purified and unpurified was estimated using JEOL JSM SEM scanning electron microscope where Energy Dispersive X ray analysis (EDX) can be performed. This data showed the elemental composition of the gelatin hydrogel samples.

3.2.2.2 Swelling Study

The gelatin hydrogel samples were cut to 1cmx1cm size and were weighed accurately (W_o). Then the samples were immersed in both water and phosphate buffer solution having pH 7.2 at room temperature to swell. Their weight (W_t) was measured every 15 minutes for one hour and then every 30 minutes for next eight hours and then every 12 hours until the weight become constant. The swelling ratios were calculated using the formula mentioned below

Swelling Ratio = $(W_t-W_o)/W_0$ x100

Where W_t is the weight of swollen gelatin hydrogel and

W_o is the dry weight of the gelatin hydrogels before swelling

3.2.2.3 In vitro degradation study

Degradation study was performed to assess the biodegradability of the gelatin hydrogel samples both purified and unpurified. Gelatin hydrogel samples were cut in 1cmx1cm size and weighed. After that these samples were soaked in phosphate buffer solution and weight changes were determined every day until the sample is degraded.

3.2.2.4 Contact angle measurement

The contact angle was measured for both purified and non purified gelatin hydrogel samples with and without cross linking of gluteraldehyde using Drop shape Analyzer DSA24 KRUSS Germany. The samples were cut in 1cmx1cm size and kept on the glass slide to analyse the samples. Distilled water was used as the water droplets for measurement of contact angle.

3.2.2.5 FTIR analysis

The chemical structure of the purified and unpurified gelatin hydrogel samples with gluteraldehyde cross linking was analysed using ALPHA Bruker FTIR. The samples were cut into 2cmx2cm size for analysis.

3.2.2.6 Rheological Measurements

Rheological measurements were done using rheometer. The parallel plate diameter was 15mm and the distance between the parallel plates were dependant on the gelatin hydrogel samples. The gelatin hydrogel samples were cut in a round shape of 2cm diameter for analysis. Storage modulus (G') and loss modulus (G'') were measured.

3.2.2.7 Scanning Electron Microscopy

Scanning electron microscopy was performed after freeze drying of the gelatin hydrogel samples.The samples were cut in 0.5mmx0.5mm size and were coated with gold sputtering to a thickness of 10nm for 10 minutes. The samples were then analysed using JEOL JSM SEM scanning electron microscope whose accelerating voltage was 10 kV and current was 5mA.

3.2.2.8 Hemocompatiblity Test

Hemocompatiblity test was performed for purified gelatin hydrogels cross linked with gluteraldehyde according to ASTM standards. The blood samples were collected and 1.5 mg of EDTA per ml of blood was added to it during the collection of blood to prevent coagulation of the blood samples. The blood sample was diluted with normal saline in the ratio of 8:10. Gelatin hydrogels were cut in 5mmx 5mm size for the hemocompatiblity test. These gelatin hydrogel samples namely 0.2M. 0.3M, 0.4M, 0.5M was added to 0.5 ml of diluted blood in a centrifuge tube which is then diluted with 9.5ml of normal saline. Positive and negative controls were prepared for the test. Positive control was prepared by mixing 0.5ml of diluted blood with 0.01 N HCl and further diluted to 10ml. HCl is used for positive control as it has the capacity to rupture the red blood cells and hence show high percentage of lysis. Negative control was prepared by diluting 0.5 ml of dilutd blood sample with normal saline to make up the volume upto 10ml. The centrifuge tubes with gelatin hydrogel samples and positive and negative control is incubated for 60min at 37˚C and it was centrifuged for 5 minutes. Further, optical density was measured using UV-visible spectrophotometer for all the gelatin hydrogel samples at 545nm.%hemolysis was calculated using following formula:

$$\%\text{hemolysis} = OD_{test}-OD_{negative}/OD_{positive}-O_{negative} \times 100$$

Chapter 4

Results and Discussion

4.1 Results

4.1.1 Preparation of gelatin hydrogels and Morphology of the gelatin hydrogels

While gelatin hydrogels were prepared using different concentration of EDTA that is 0.2M, 0.3M, 0.4M and 0.5M, all concentrations lead to formation of gel network. 0.2M and 0.3M concentration of EDTA lead to formation of a very loose structure of the gel whereas 0.4M and 0.5M concentration of EDTA formed a comparatively firm structure (Figure 18).

When after using EDTA these purified gelatin hydrogel samples were cross linked with different concentration of 25% aqueous gluteraldehyde that is10µl, 20µl, 30µl, 40µl and 50µl and gel was only formed with 10µl, 20µl, 30µl concentration (Figure 19). At higher concentration gel was formed in aggregating form in the beaker before casting it to the petri dishes.

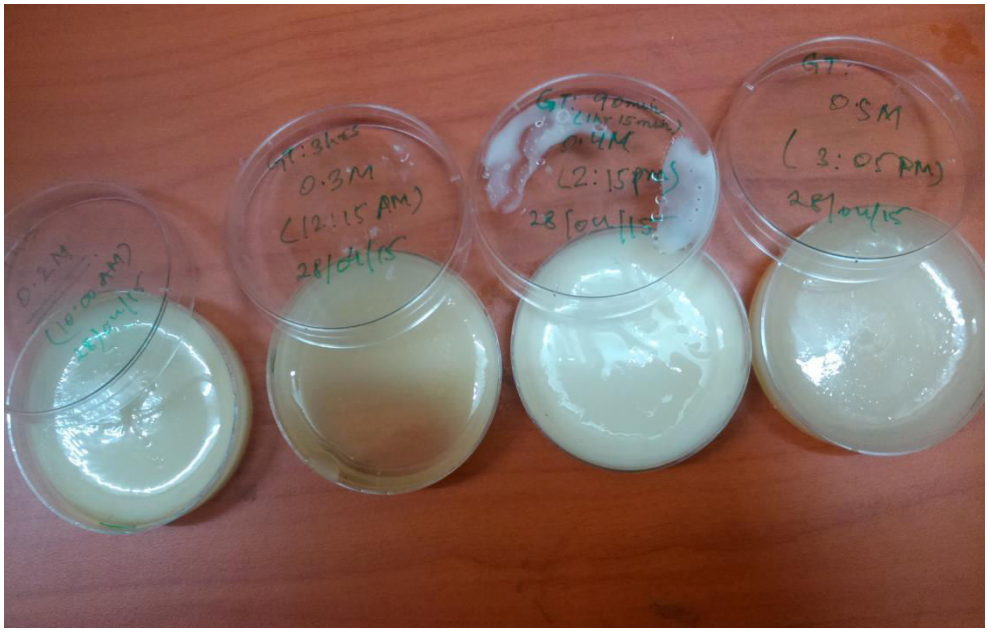


Figure 18: Purified Gelatin Hydrogels with EDTA

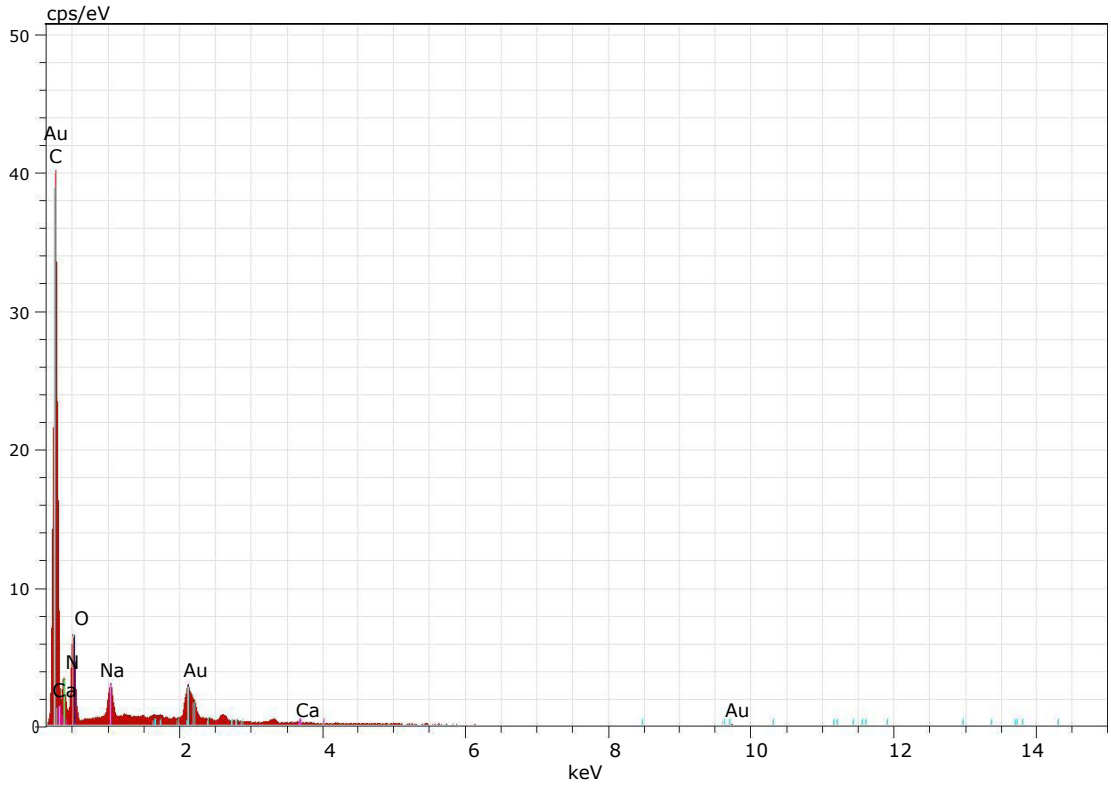
Figure 19: Purified Gelatin Hydrogel after Crosslinking with Gluteraldehyde

4.1.2 Ion measurement

The divalent ion concentration of purified gelatin hydrogels were analysed using the EDX spectrum and it is concluded that the concentration of all the divalent ions are shown to be in a very low concentration after addition of different concentration of EDTA solution. The concentration of calcium ion was shown as 0% (Table 4) after addition of EDTA to the gelatin hydrogel sample when compared with unpurified gelatin hydrogel where concentration of both calcium and other divalent ions are seen to be higher in the concentration, calcium concentration shown as 0.24% (Table 3). Chemical composition of both unpurified and purified gelatin hydrogel samples and EDX spectrum is shown below:

El	AN	Series	Net unn. [wt.%]	C [wt.%]	norm.C [at.%]	Atom. C [wt.%]	Error (1 Sigma) [wt.%]
C	6	K-series	84738	51.97	51.97	57.85	5.94
O	8	K-series	14908	24.00	24.00	20.05	3.21
N	7	K-series	6996	21.93	21.93	20.93	3.27
Na	11	K-series	6280	1.86	1.86	1.08	0.15
Ca	**20**	**K-series**	**457**	**0.24**	**0.24**	**0.08**	**0.04**
Au	79	M-series	12536	0.00	0.00	0.00	0.00
		Total:		100.00	100.00	100.00	

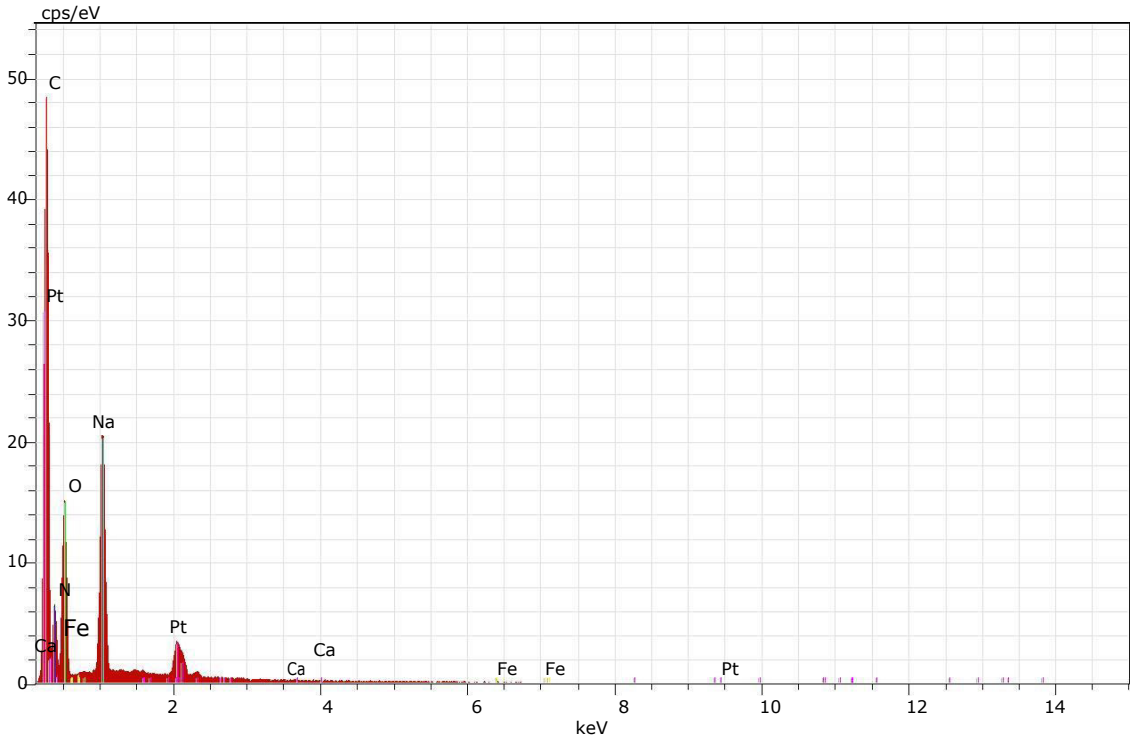
Table 3: Chemical composition of unpurified Gelatin hydrogels



Graph 1: EDX Spectra of Unpurified Gelatin hydrogel

El	AN	Series	Net unn. [wt.%]	C norm.C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
C	6	K-series	69349	42.27	42.27	49.38	4.89
O	8	K-series	24584	27.95	27.95	24.51	3.53
N	7	K-series	9418	20.28	20.28	20.31	2.89
Na	11	K-series	38010	9.47	9.47	5.78	0.62
Fe	26	K-series	26	0.03	0.03	0.01	0.03
Ca	**20**	**K-series**	**0**	**0.00**	**0.00**	**0.00**	**0.00**
Pt	78	M-series	9926	0.00	0.00	0.00	0.00
Cu	29	K-series	0	0.00	0.00	0.00	0.00
		Total:	100.00	100.00	100.00		

Table 4: Chemical composition of Purified Gelatin Hydrogels

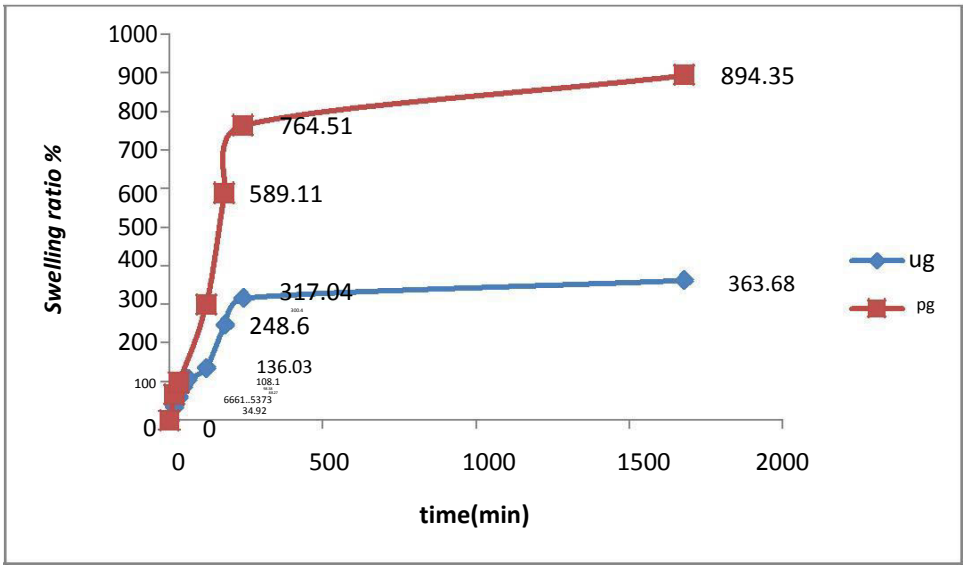


Graph 2: EDX spectrum of Purified Gelatin Hydrogel

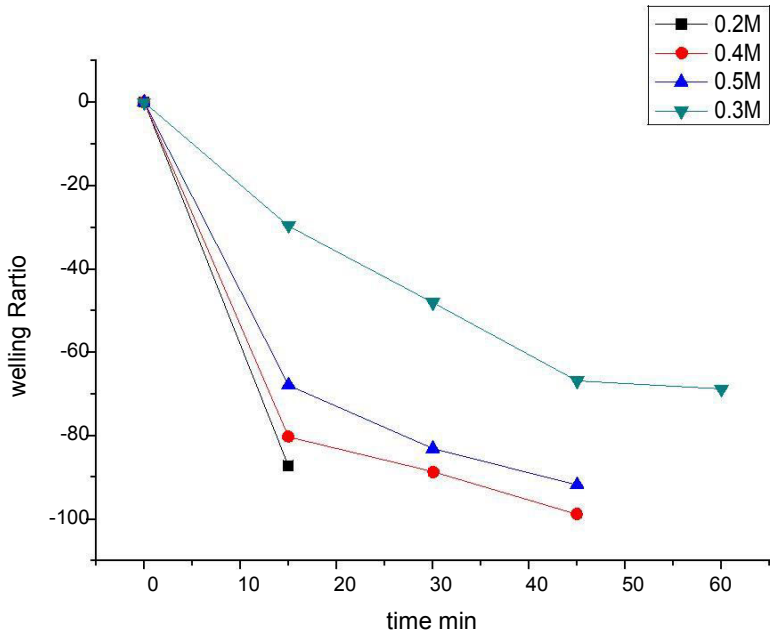
4.1.3 Swelling Studies

Swelling Ratios are used as a method to characterize water absorption and stability of biomaterials(Peng, Martineau, & Shek, 2007). The swelling behaviour of purified and unpurified gelatin hydrogels without crosslinking was analysed and and was found to be 894.35% and 363.68% (Graph 3). Swelling behaviour of purified samples without and with cross linking was also analyzed in PBS at 37°C and it is found that both purified gelatin hydrogels with and without crosslinking showed lower swelling ratio when compared to that in deionized water (Graph 4). After crosslinking swelling ratio decreased due to dense network formation. Swelling ratio of purified gelatin decreased in PBS as it has very high ionic strength compared to water leading to loss of water from the purified gelatin hydrogels. This phenomenon does not allow the absorption and hence decreases the weight of the gelatin

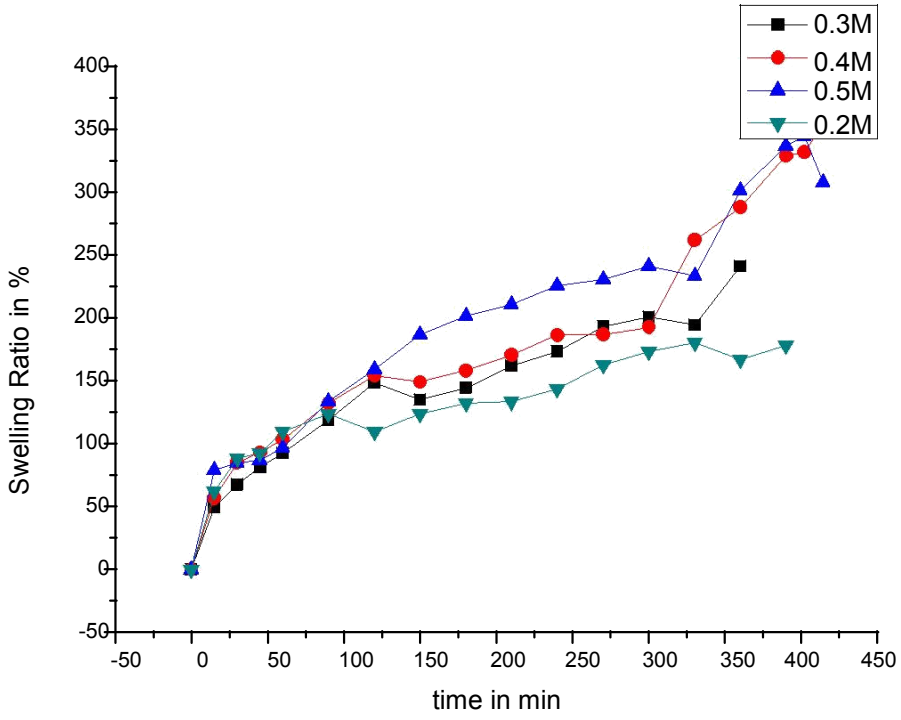
hydrogels (Figure23). In case of purified gelatin with gluteraldehyde, hydrogels swelled in PBS due to presence of cross linking agent present in the hydrogels (Graph 5).



Graph 3: Swelling Graph of Purified and Unpurified gelatin hydrogel in water



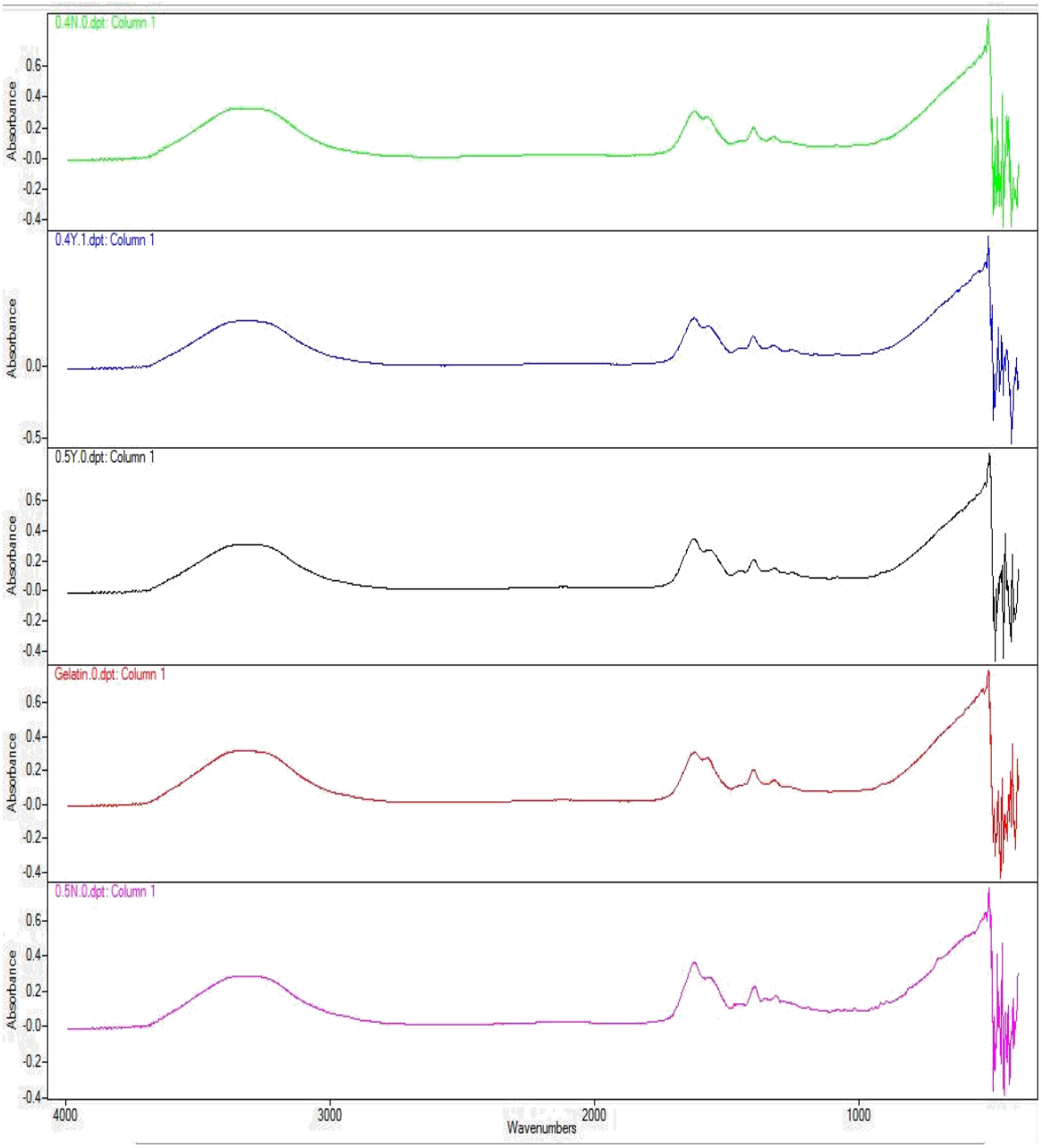
Graph 4: Swelling Graph of Purified Gelatin Hydrogel without Crosslinking in PBS



Graph 5: Swelling Graph of Purified Gelatin Hydrogel with Crosslinking in PBS

4.1.4 FTIR characterization

FTIR data analysis was performed using software e spectrum. This spectrum is analyzed to compare peaks between unpurified gelatin hydrogels, purified gelatin hydrogels and purified gelatin hydrogels with gluteraldehyde crosslinking. By comparing these samples we can infer that there is no shift in the peaks and hence there is no change in the chemical structure of gelatin hydrogels (Graph 6). Hence the addition of EDTA did not affect the structure of the gelatin gel network.



Graph 6: FTIR spectra of Unpurified and Purified Gelatin Hydrogel samples

4.1.5 SEM images

SEM images of purified gelatin hydrogel samples before cross linking and gelatin hydrogel samples after cross linking was analyzed. SEM images of purified gelatin hydrogel samples which are not cross linked showed larger pores (Figure 21) in them whereas those purified samples which are cross linked using gluteraldehyde showed denser network (Figure 20).

Also the stability if gelatin hydrogels were determined by swelling the gelatin hydrogels and studying the morphology. The unpurified gelatin hydrogels swell from between first making it very unstable whereas the purified gelatin hydrogels which were cross linked swell uniformly showing the stability of the gelatin gel network (Figure 22).

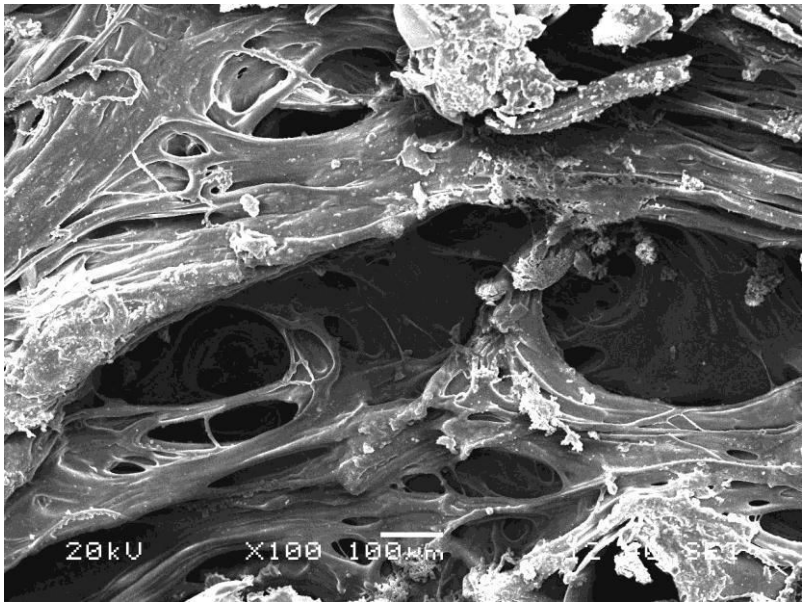


Figure 20: Purified gelatin hydrogels with crosslinking

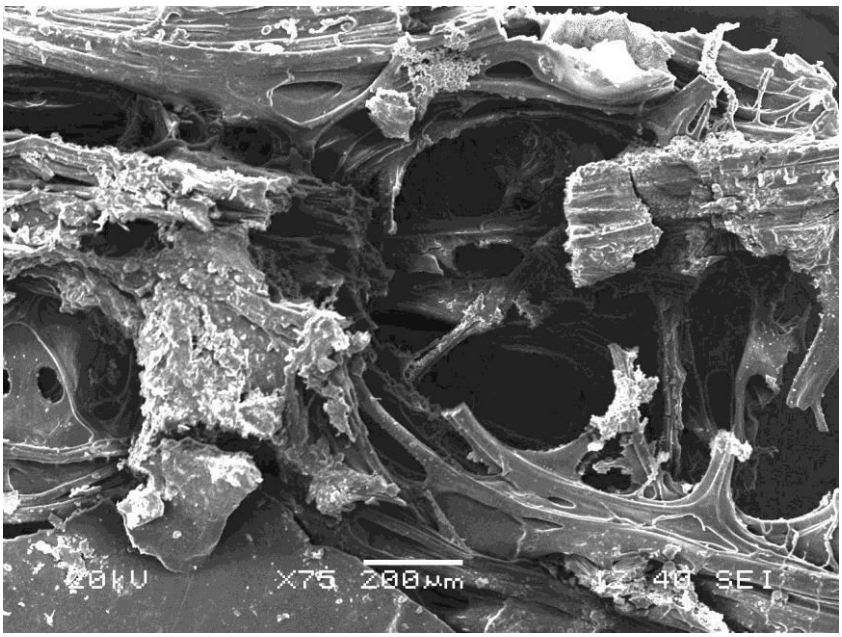


Figure 21: purified gelatin hydrogels without crosslinking

Figure 22: Stable Purified gelatin hydrogels with gluteraldehyde crosslinking

4.1.6 In vitro degradation

In vitro degradation studies were performed on purified gelatin hydrogels with and without crosslinking with gluteraldehyde in PBS at 37°C. Purified gelatin hydrogels without gluteraldehyde degrade in 2 hours whereas gelatin hydrogels with gluteraldehyde were very stable and they remained in PBS for 8 days and then degraded which showed that these hydrogels have excellent stability at physiological conditions.

4.1.7 Contact Angle Measurement

Contact angle depicts the wet ability of the biomaterial. Contact angle was measured for purified gelatin hydrogels with and without gluteraldehyde crosslinking where purified gelatin hydrogel without gluteraldehyde showed angle of 78.6° and purified gelatin hydrogel with gluteraldehyde showed the angle of 55.83° (Figure 23). Both the readings depicts that the gelatin hydrogels are hydrophilic in nature and hence can allow cell attachment easily.

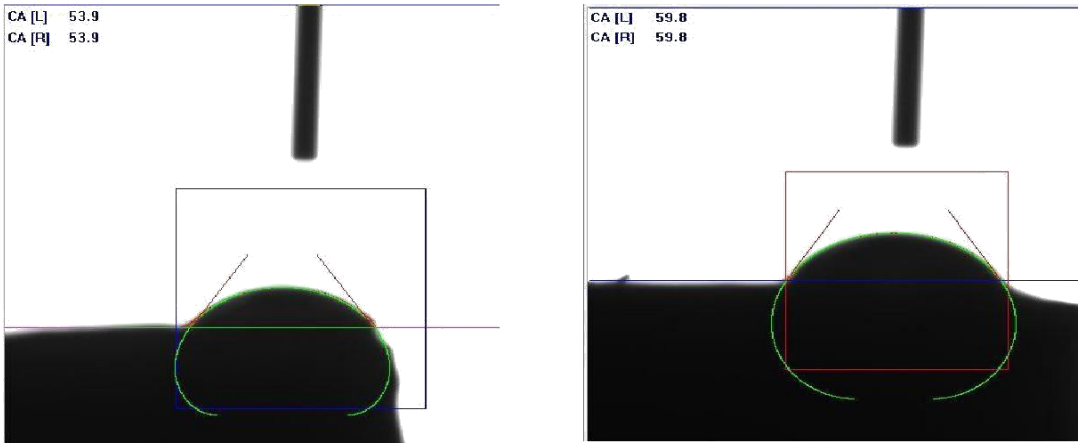
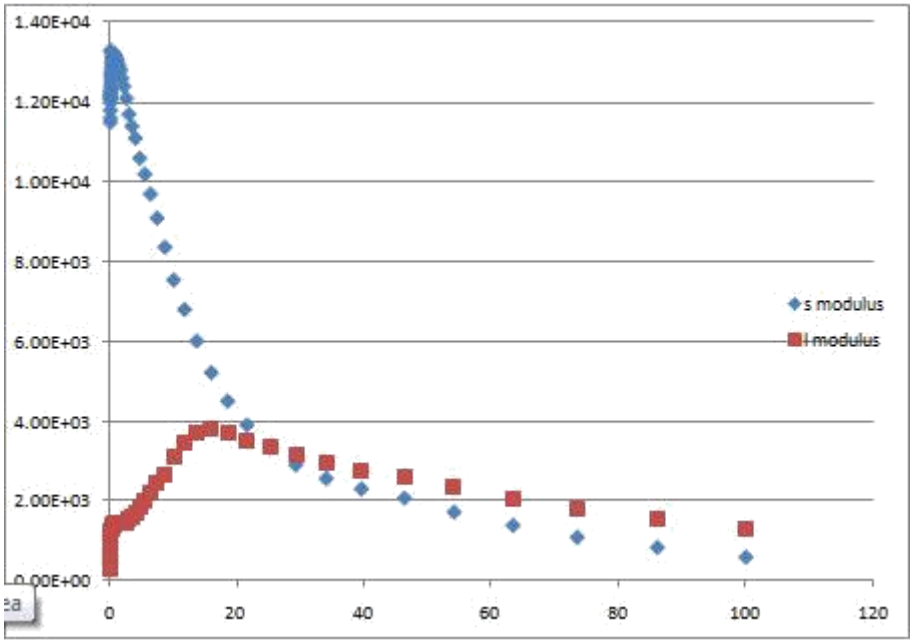


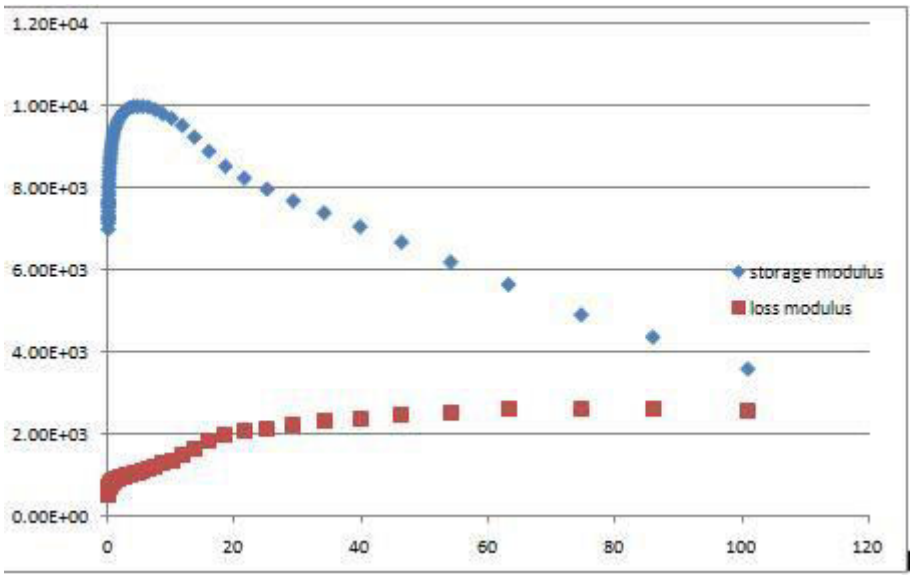
Figure 23: Contact Angle measurement a) Purified gealtin hydrogel without gluteraldehyde b) purified gelatin hydrogel with gluteraldehyde

4.1.8 Rheology measurements

Rheological measurements of both purified gelatin hydrogels with and without cross linking always depict the viscoelasticity of the gel system. Higher values of storage modulus in both the graphs show higher elasticity of the gelatin hydrogel. The point where both storage modulus and loss modulus intersect is always known as gel point. In the graphs below elasticity of the gel starts decreasing i.e elasticity decreases and loss modulus increases i.e viscosity of the gelatin hydrogel increases when strain is applies on both purified gelatin hydrogel with and without cross linking with gluteraldehyde.



Graph 7: Storage and loss modulus of purified gelatin without cross linking



Graph 8: Storage and loss modulus of purified gelatin with cross linking

4.1.9 Hemocompatiblity test

Hemocompatibilty of the purified gelatin hydrogel with gluteraldehyde crosslinking was performed. If the % hemolysis comes less than 5 then the biomaterials are considered highly

hemocompatible, if less than 10 hemocompatible and if it more than 10 biomaterials are not considered to ne hemocompatible in nature. This test shows the ability of red blood cells to rupture under osmotic stress conditions where saline act as a hypertonic solution. Due to high stress haemoglobin comes out of the cell and leads to measurement of % hemolysis.% hemolysis data is collected and it is concluded that gelatin hydrogel samples are highly hemocompatible in nature.

S.no	Sample	OD at 545nm	% hemolysis
1.	Positive control	0.324	-
2.	Negative control	0.104	-
3.	0.2M	0.096	0.04
4.	0.3M	0.105	0.07
5.	0.4M	0.102	0.05
6.	0.5M	0.106	0.043

Table 5: % hemolysis of each gelatin hydrogel Samples with gluteraldehyde

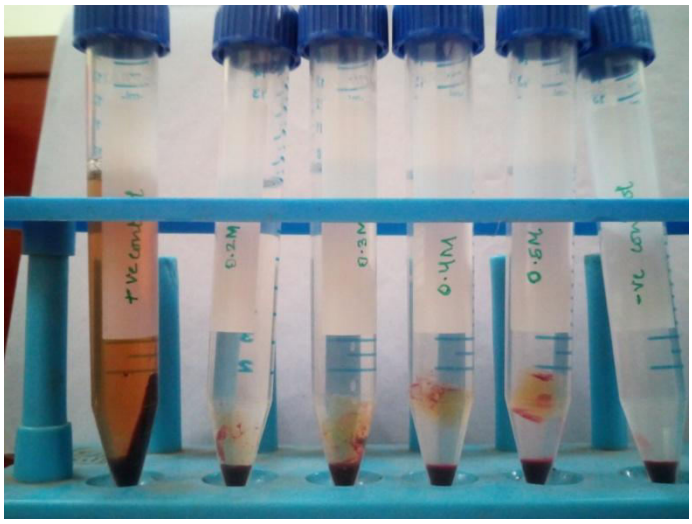


Figure 24: Hemocompatiblity test performed with cross linked purified gelatin hydrogels

4.2 Discussions

Gelatin hydrogels contains many divalent ions such as calcium, copper, iron due to its denaturation from the collagen. These divalent ions play a very important role in the formation of gelatin gel network. These divalent ions form ionic bonds with carboxylic acid (Figure 25). Gelatin aggregation is accelerated mainly due to presence of these divalent ions in the gelatin hydrogels. Gelatin gels are mainly formed due to physical crosslinking. During the gelation positively charged carboxylic acid interacts with negatively charged amine groups with the help of electrostatic interactions. The gelatin hydrogels are highly hydrophilic in nature thereby absorbing large amount of water through the hydrogen bonds formed between water molecules and amino groups and carboxylic groups. When cross linked with gluteraldehyde amide bonds are formed between carboxylic acid and amino groups.

When the gelatin hydrogel is chelated with EDTA it diminishes the activity of all divalent ions present in the gelatin network thereby rendering the carboxylic acid group free in the gelatin network. This was clearly depicted by EDS spectra of the purified gelatin hydrogel where the concentration of the divalent ion were present in very small amount and calcium divalent ion concentration was shown to be 0%.

As more number of carboxylic acid groups are free in the gelatin network this makes the gelatin network more hydrophilic in nature. This is depicted by contact angle measurement data which shows high hydrophillicity nature of purified gelatin hydrogels without and with cross linking agent gluteraldehyde. Hence more amount of water is absorbed in the purified gelatin network as more hydrogen bonds are formed after chelation due to free carboxylic acid present which is clearly depicted by the swelling studies. Also less aggregated and loose structure gel network is formed after chelation due to higher absorption of water and more

number of free carboxylic acid. This leads to easy penetration of gluteraldehyde leading to formation of more dense structure which is depicted by SEM images. This cross linking agents leads to cross linking of more carboxylic acid and amino groups in the gelatin network when compared to the unpurified gelatin hydrogels.

The swelling behaviour of unpurified gelatin hydrogels is affected by the hydrophilic groups present in the polymer. After chelation stronger ionic bonds are broken and and is replaced by weaker interaction which made purified gelatin hydrogels to absorb more amount of water. When swelled in water purified gelatin network did not swell due to higher ionic strength of PBS and Donnan effect water comes out of the purified gelatin network and leads to decrease in the swelling ratio. Whereas when cross linked with gluteraldehyde swelling ratio decerased due to formation of dense gelatin network. This dense network is formed due to presence of more amount of free carboxylic acid interacting with amino groups within the gelatin network

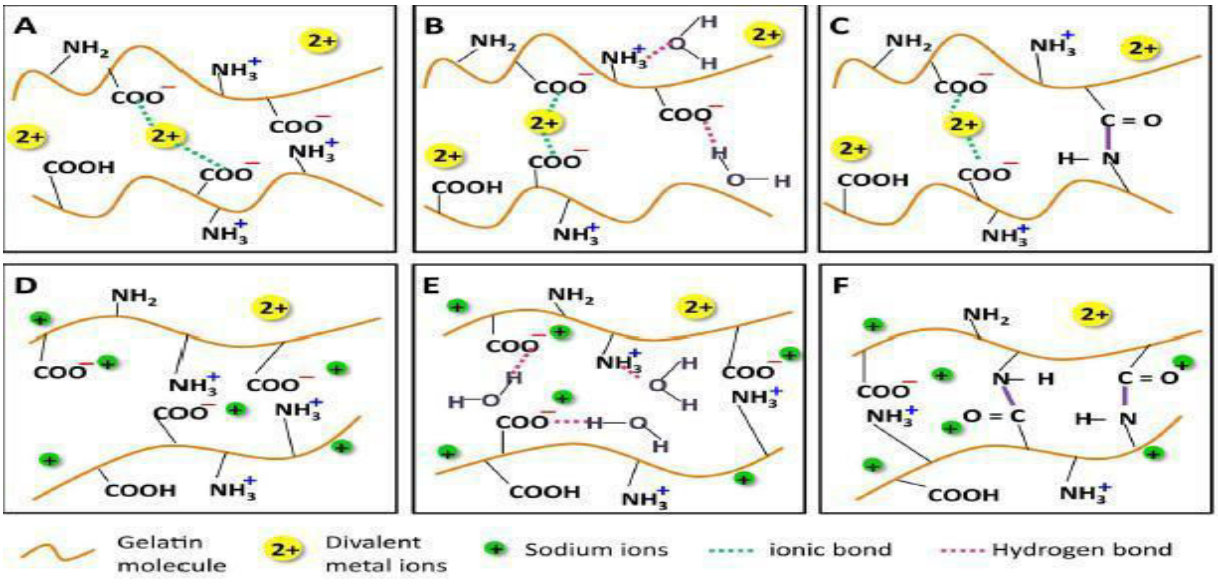


Figure 25: Interaction between gelatin and divalent ions before and after the divalent removal

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