“PHYSICO CHEMICAL CHARACTERIZATION OF CHITOSAN BASED SCAFFOLD AS WOUND HEALING BIOMATERIAL”

A THESIS SUBMITTED

BY

DIVYA M R
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THIRUVANANTHAPURAM – 695 011

DECLARATION

I, **DIVYA M R**, hereby declare that I had personally carried out the work depicted in the thesis entitled "PHYSICO CHEMICAL CHARACTERIZATION OF CHITOSAN BASED SCAFFOLD AS WOUND HEALING BIOMATERIAL", under the guidance of **Dr. Rekha M.R,** Division of Biosurface Technology**,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**PHYSICO CHEMICAL CHARACTERIZATION OF CHITOSAN BASED SCAFFOLD AS WOUND HEALING BIOMATERIAL**" submitted by **Divya M R** in partial fulfillment for the degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Division of Biosurface Technology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram.

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Divya M R

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ABBREVIATIONS

CS	Chitosan
CSDTPA	Chitosan – dithio dipropionic acid
DD	Degree of Deacetylation
DMSO	Dimethyl sulphoxide
DTPA	Dithio dipropionic acid
DTT	D, L-dithiothreitol
EDC	1-Ethyl-3- [3-dimethyl amino propyl) carbodiimide hydrochloride
FBS	Fetal Bovine Serum
FTIR	Fourier transform Infrared spectroscopy
HCl	Hydrochloric acid
MEM	Minimal Essential Medium
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
PBS	Phosphate Buffer Saline
WVTR	Water Vapour Transmission Rate

SYNOPSIS

Wound is a scratch or injury to our skin. Our body has a complex system and have cascade of events to heal the wound. There are three stages in wound healing and they are overlapped. The stages of wound healing are inflammation, proliferation and maturation. In wound types chronic wounds are challenging to treat. The current treatment methods to treat wound is antibiotic delivery through oral rout or intravenously. Its disadvantage is low bioavailability and can cause toxic effects. To overcome the systemic toxicity and low bioavailability of therapeutic molecule biomaterials are used as a scaffold for local delivery of the drugs.

This thesis has been divided into four chapters; the first chapter briefly discusses the wound healing and current treatment strategies, about chronic wounds, advanced wound care materials, chitosan in wound healing, drug delivery in wound healing, redox sensitive materials in drug delivery, and effect of free radicals on disulphide bonds, hypothesis and objectives of the study.

The second chapter includes a literature review. The third chapter deals with the materials and methods employed in the study, including fabrication of chitosan based CSDTPA scaffolds in three different concentrations. The scaffolds are characterized for its physico-chemical properties and *in vitro* cytotoxicity. The physico-chemical characterization was evaluated using techniques like, Fourier Transform IR Spectroscopy (FTIR), Molecular weight determination using Ubbelohde viscometer, determining the degree of deacetylation. The suitability of CSDTPA to act as a scaffold was analysed by doing porosity analysis, swelling Analysis, redox responsiveness of

CS-DTPA, Determining the weight loss percentage, and water vapour transmission rate. The drug loading and releasing ability of the scaffold was analysed in *in vitro* drug release study which needs further investigation. The cytotoxicity of the scaffold was also analysed in *in vitro* cytotoxicity-MTT Assay.

Fourth chapter describes the result and discussion of the study. CSDTPA200 was chosen as the optimized scaffold for further study. Cell viability studies are done in this scaffold to check the suitability. The Fifth chapter gives the conclusion that as of now CSDTPA200 scaffold was chosen to be the one for further analysis as it shows stability in all pH.

CHAPTER 1

INTRODUCTION

1.1 Background

The current strategy to treat chronic wounds is to deliver antibiotics orally or intravenously which is of low bioavailability and cause adverse toxic effects. This limitation is prominent especially in diabetic wounds, wounds derived from removal of malignant or tumorous tissue, infected non-healing and burn wounds. The blood vessels are compromised in these cases owing to chronic nature of the wound and this leads to poor delivery of drugs from circulation to the site. To overcome this poor bioavailability often high doses of drugs are administered which leads to systemic toxicity and multiple organ failure. Recently local delivery of therapeutic molecules using biomaterials to the site of need is gaining attention and is being investigated vigorously. The scaffold based drug delivery can be regulated to have a controlled and sustained release of drugs. It is of immense potential in wound management and regenerative medicine.

1.2 Wound healing and current treatment strategies

Wound healing process is an intricate and essential regulated sequence of several well-organised biochemical and cellular phenomena to restore the integrity of the skin. During the wound healing process, wound progresses within three differentiated, though overlapping stages: inflammation, proliferation (neo-angiogenesis, granulation, re-epithelialization), and maturation (extracellular matrix [ECM] remodelling).

1.3 Chronic wounds

Among the wounds one of the most challenging to treat are the chronic wounds. The prevalence rate of chronic non-healing wounds is increasing considerably with increase in diseases such as diabetes, geriatric population, etc. Diabetes is a chronic metabolic disease and the current diabetic population comes to 463 million and is expected raise to 578 million by 2030 (Saeedi et al., 2019). As per the reports about 20% of the diabetic patients develop wounds of which foot ulcers is the most common and the risk of lower extremity amputations is 17–40 times higher in diabetic patients than others (Paul et al., 2020).

In chronic non-healing diabetic wounds the healing gets stalled at inflammatory phase. The main characteristics of inflammatory phase are that pro-inflammatory macrophages are active leading to elevated levels of inflammatory cytokines, reactive oxygen species and proteolytic enzymes (Hu et al., 2016). Proteases degrade growth factors and newly synthesized components of the extracellular matrix (elastin and collagen) while the elevated levels of free radicals (super oxide (O_2-), hydroxyl radical (OH^-) and nitric oxide (NO) and their reaction products oxidize biomolecules which leads to activation of the inflammatory systems thereby creating an impairment of the healing of chronic wounds.

1.4 Advanced wound care materials

Recently, advanced wound care materials started taking place of conventional wound care materials. Advanced wound care materials have proved to have a positive impact on various sectors of wound healing. Natural/synthetic polymer hybrids, combination of biopolymers, with better properties that are crucial for wound healing may provide opportunities to synthesize matrices that can provide better care. In the case of advanced wound care materials it is mainly various forms of biomaterials which plays a significant role for developing novel wound dressings. These biomaterial dressings can be designed to have intrinsic wound healing activity or/and may serve as a support for the release of bioactive compounds.

Molecules with high radical-scavenging capacity have been reported to facilitate wound-healing. A relatively new approach to wound healing involves the use of polymeric wound dressings to deliver various pharmacological agents that can take active part in one or more stages of the wound healing process. The activities of these compounds together with the physical characteristics of the dressing can enhance the wound healing rate.

1.4.1 *Chitosan in wound healing*

Chitosan is a natural polysaccharide found in the cell walls of fungi, crustaceans and insects. It is cationic in nature and has good biocompatibility and sensitivity to pH (Yanat et al. 2021).

Chitosan has shown solubility in acids like acetic acid, lactic acid, and malic acid, etc. Chitosan based materials are used in biomedical field due to its properties like biodegradability and biocompatibility. Chitosan stimulates hemostasis and increases tissue regeneration in biomedical research natural products are used more than synthetic ones. Chitosan is having biodegradability and used in wound healing studies due to its hydrophilic nature and structurally similar to glycosaminoglycan the monomeric unit N-acetylglucosamine of chitosan is found in hyaluronic acid, which is important in wound repair (Shakeel et al. 2016).

A good chitosan scaffold has stability and porous nature which can be fabricated by doing surface modification and lyophilisation. When chitosan is conjugated with other materials for modification, the properties of chitosan may get affected. So there is a need for evaluation of the conjugates and material used, to make sure they are good in quality and biocompatible. So they can be used in wound management. Chitosan can be easily made in to different forms like, hydrogels, scaffolds, and films, etc. Because of properties like these chitosan has large number of applications in wound healing and drug delivery (Anithaa et al., 2014).

1.4.2. *Drug delivery in wound healing*

Delivery of new drugs loaded in scaffolds for wound management needs to be investigated. Comparing local and systemic drug delivery, in systemic drug delivery IV injection or oral route is commonly used for drug administration. Unfortunately, this requires higher dosing of drugs to achieve the therapeutic effect in the affected tissue/organ. So, there is a potential chance for harmful side effects in non- target tissues. Considering the risks of systemic toxicity and less expected drug delivery to the target site, clinical studies are focusing on localized delivery of drugs for wound healing. Localized drug delivery allows agreeable self- administration for patients and avoids issues with GI absorption and hepatic first pass metabolism, thereby improving bioavailability and maintenance of drug concentration within the therapeutic window. Local delivery allows passage of the largest fraction of drug molecules to the target area, while maximizing therapeutic potential and reducing systemic toxicity (Wittam et al., 2014).

1.5 Redox sensitive materials in drug delivery

Recently, numerous polymers with bio-responsive functions sensitive to various intracellular signals, such as acidic pH, temperature, and redox potential, have been developed and used as intracellular delivery systems. Redox-sensitive micelles rely on disulfide bonds either in the matrix crosslink, or in auxiliary chains for triggered release. These bonds remain highly stable when placed in extracellular environments which are highly oxidizing. However, they are rapidly broken through thiol–disulfide exchange reactions in the cytosol containing two to three orders-of-magnitude greater levels of glutathione (GSH) (approximately 2–10 mM) than those found in extracellular fluids (approximately 2–20 μM) (Meirong et al., 2016).

Disulfide bond plays an important role in various fields, for example, biochemical processes, industrial and pharmaceutical chemistry, bioconjugates, peptide-mimetics and self -assembled monolayers (SAMs), etc. In the past decades, drug delivery systems (DDS) have been widely investigated and disulfide-based oxidation-reduction responsive DDS is the most promising one. Disulfide bonds have no physiological toxicity and remain stable in human body, and can be broken into a reduced form of glutathione (GSH) *via* the thiol-disulfide exchange reaction.

1.6 Effect of free radicals on disulphide bonds

Adhikari et al. (2020) recently reported the effect of hydroxyl radicals in cysteine disulfides and a two-step reaction mechanism was proposed. The observation from experimental and theoretical analysis was that the reactions of •OH and cysteine disulfide compounds produce four types of sulfur species, namely –SOH, –SO•, –SH, and –S•. All these are important in the sulfur redox cycle. They also identified that among–SOH is a potent scavenger for peroxyl radicals via H atom transfer. The study concluded that by reacting cysteine disulphide with •OH can lead to formation of a potent antioxidant in nonpolar environment. Thus disulfides can be made to be more actively involved in the antioxidant groups so as scavenge the elevated levels of reactive oxygen species.

1.7 Hypothesis

By introducing disulphide bonds in chitosan antioxidant property can be imparted and also its drug delivery potential may be improved in chronic wound environment.

1.8. Objectives

Chitosan based scaffold with disulphide linkages will be developed for localised therapeutic delivery applications.

1. Preparation of derivatives of chitosan for scaffold development.
2. Physico-chemical characterisation of the chitosan conjugates- FTIR, chemical analysis and cytocompatibility studies.
3. Scaffold development and its physico-chemical characterisation.
4. Drug loading and release.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Wound Healing

Skin act as a protecting cover to internal organs from foreign environmental hazards. Skin has outstanding regenerative properties and injuries or wounds are healed through a highly organized cascade of physiological events. Notable efforts have been done to developing alternate therapies that could replace the regenerative properties of the natural skin. Different types wound care systems are developed to treat the wound and enhance the healing process (Saghazadeh et al., 2018). Normal wound healing have four different but overlapping stages, that are hemostasis, inflammation, proliferation, and remodelling (Negut et al., 2020). Neutrophils are the most abundant cells in the early stages of healing. As they degranulate and die, macrophages predominate, accumulating at the wound site following recruitment from the circulation and from the resident population. Macrophages are vital for normal healing and if their infiltration is prevented, healing is impaired.

Increased macrophage infiltrate at the wound site can also perturb the normal wound healing process, contributing to the development of non-healing wounds (Loots et al. 1998).

Moreover, macrophage conversion from a pro-inflammatory to an anti-inflammatory phenotype is critical to wound resolution. If this phenotypic conversion does not occur or reach completion, it may lead to the development of chronic wounds (Snyder et al. 2016).

2.2 Stages of Wound Healing

Wound repair is classically simplified into four main phases: haemostasis, inflammation, proliferation and dermal remodelling (Broughton et al. 2006).

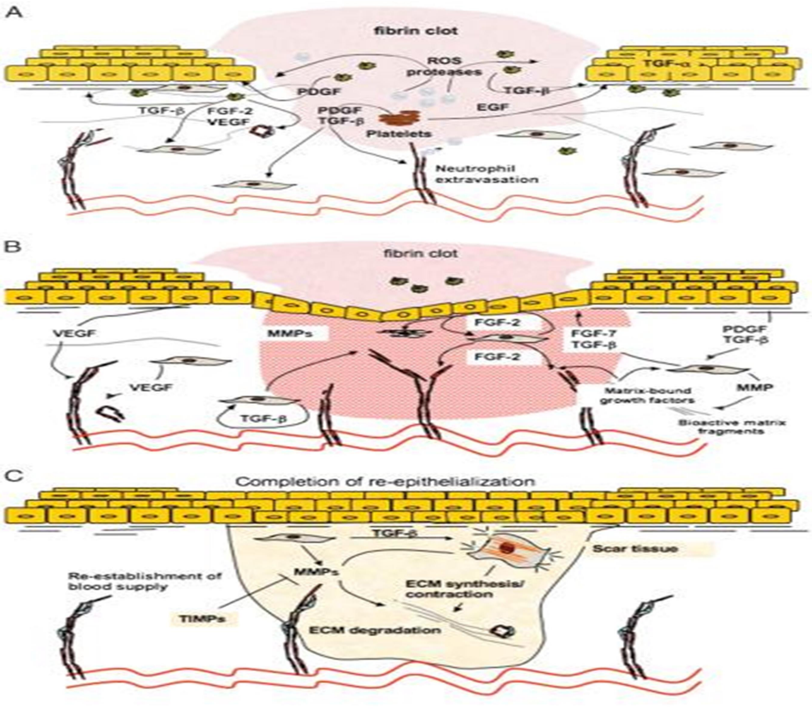


Figure.1. Stages of wound healing (doi: 10.1097/01.ASW.0000416006.55218.d0)

2.2.1 *Haemostasis*

Within seconds of skin injury, vasoconstriction occurs to reduce blood loss. As blood spills into the wound area, platelets come into contact with and adhere to the wall of the injured blood vessels and to the exposed collagen within the extracellular matrix. This triggers platelets to release cytokines, growth factors and numerous pro-inflammatory mediators resulting in platelet aggregation and triggering the intrinsic and extrinsic coagulation pathways, leading to fibrin clot formation (Pauline et al. 2010).

2.2.2 *Inflammation*

Tissue injury and the activation of the coagulation cascade stimulate the release of vasoactive cytokines such as prostaglandins, histamine and other amines from the granules released by mast cells as a response to injury, which results in increased local vasodilation and capillary permeability.

2.2.3 *Proliferation*

During the proliferative phase the wound defect is filled with highly vascular connective tissue, commonly referred to as ‘granulation tissue’. This process is instigated at the point of wounding, when as a result of loss of vascularity in the wound bed the wound environment has a low pH, reduced oxygen tension and increased lactate; this initiates the release of vascular endothelial growth factor (VEGF), basic fibroblast factor (bFF) and TGF-b, all of which activate neovascularization or angiogenesis.

2.2.4 Epithelialization

In this final stage of visible wound healing, epithelial cells migrate from the wound edges to resurface the wound defect. In wounds healing by primary intention epithelialization occurs concurrently with connective tissue deposition. In wounds healing by secondary intention this process is delayed until the wound defect is filled with granulation tissue. Epithelial cells can only migrate over a moist, vascular wound surface; they are inhibited by a dry or necrotic wound surface.

2.2.5 Remodelling phase

This may extend to a year or more, during which fibroblasts regulate the process of wound matrix breakdown by matrix metalloproteinase (MMPs) and synthesis of new extracellular matrix. This slow process increases the tensile strength of the wound, but scar tissue is never more than 80% of the tensile strength in unwounded tissue.

2.3. Chronic Wounds

Chronic wounds represent a major health burden and drain on resources. Recent advances in our understanding of chronic wound biology have led to the development of several new treatments that offer renewed hope to patients with ulcers and other chronic wounds (Harding et al. 2002). Chronic wounds include, but are not limited, to diabetic foot ulcers, venous leg ulcers, and pressure ulcers (Frykberg and Banks, 2015).

A very large percentage of chronic wounds fall into 3 main categories—pressure sores, diabetic ulcers, and venous ulcers—with a small fourth set of ulcers secondary to ischemia (arterial insufficiency, radiation changes). Although they have very different underlying etiologies, and the tendency has been to emphasize their differences, they do have some important causative factors in common (Mustoe et al., 2004).

2.4 Materials Used For Wound Healing

Different types wound care systems are developed to treat the wound and enhance the healing process. Drug delivery systems in the range of nano, micro, and macro scales can increase half-life, improve bioavailability, optimize pharmacokinetics, and decrease dosing frequency of drugs. Macro-system delivery by scaffolds is required to wound healing as they can store therapeautics for delivery. They can protect the wounds as wound dressings. Therefore, drug-incorporated scaffolds are promising for speeding up the healing process of chronic wounds (Kim et al., 2018).

Chitosan have a favorable impact on the different stages of wound healing, such as fibrous tissue formation, collagen synthesis and contraction, in faster healing. Faster healing of wounds by chitosan has been credited to its stimulating activity and/or its capacity to stimulate fibroblast proliferation, resulting in the progression of wound healing. It was reported that chitosan stimulated the migration of polymorphonuclear as well as mononuclear cells and accelerated reepithelialisation and normal skin regeneration (Singh et al., 2017).

2.4.1 *Chitosan-based delivery systems for wound healing*

Lee et al. (1997) reported the synthesis of poly (ethylene glycol) and β-chitosan which was cross-linked with a uv-active agent to give semi-IPN hydrogels. Drug-containing poly (ethylene glycol)/chitosan microspheres with glutaraldehyde cross-linking have also been reported. Charernsriwilaiwat et al., (2012) reported in their study chitosan-EDTA based nanofibrous mats loaded with lysozyme showed a potential for wound healing.

Mohamoud et al., (2016) assessed norfloxacin loaded chitosan scaffold for wound healing. In this study the chitosan is combined with collagen to load the drug. The results shows mechanical strength is depend on the type of chitosan used. The scaffold has high water absorption capacity and biodegradability. Chitosan mixed with HCl scaffolds had good bio adhesive property, and the scaffolds shown 100% drug release.

2.5 Disulfide Bond in Drug Delivery

Wu et al., (2009) reported that thiol-modified chitosan cross linked with the synthetic hydrogel ECM showed in vitro release of insulin and BSA release could be controlled by adjusting the composition, loading and disulfide bond contents. In vitro cell compatibility results were indicated that the hydrogels are biocompatible. This shows that disulphide- cross linked chitosan hydrogels have good biocompatibility and can be used in drug delivery, tissue engineering and cell culture applications.

Wu et al., (2018) synthesised a non-toxic, injectable, and thermo-sensitive NIPAAm-g-chitosan hydrogels with thiol modification. It showed good biocompatibility and biodegradability. By incorporating thiol side chains in to chitosan by N-acetyl-cysteine/EDC conjugation the mechanical properties were improved. With disulfide bond cross- linking thiol modified NIPAAm-g-chitosan maintained their biocompatibility without cytotoxicity in mesenchymal stem cells, fibroblasts, and osteoblasts.

Adhikari et al. (2020) recently reported the effect of hydroxyl radicals in cysteine disulfides and a two-step reaction mechanism was proposed. The observation from experimental and theoretical analysis was that the reactions of •OH and cysteine disulfide compounds produce four types of sulfur species, namely –SOH, –SO•, –SH, and –S•. All these are important in the sulfur redox cycle. They also identified that among–SOH is a potent scavenger for peroxyl radicals via H atom transfer. The study concluded that that by reacting cysteine disulphide with •OH can lead to formation of a potent antioxidant in nonpolar environment. Thus disulfides can be made to be more actively involved in the antioxidant groups so as scavenge the elevated levels of reactive oxygen species.

Liu et al., (2017) has reported in their study using Pluronic F127 an amphiphilic triblock copolymer which shows a potential for drug delivery as it can incorporate drugs which are hydrophobic and arrange them into nano size micelles, but it lacks stimuli responsive behavior. To compensate this they synthesized modified F127 with alpha-tocopherol through redox sensitive disulfide bond between them. The conjugate showed stability at relatively low critical micelle concentration and also shown sensitivity to the intracellular environment. They used 3,3′-dithiodipropionic acid as redox sensitive agent. The modified F127-tocopherol ruptured in the presence of glutathione as a result of redox reaction. These studies showed the potential application of the F127-tocopherol to be a stable and safe formulation for drug delivery system.

Guan et al., (2020) synthesized a glycyrrhetinic acid –PEG polymer conjugated with 3, 3’-dithiopropionic acid via amide and esterification reaction which targets the liver. Results showed that the drugs encapsulated in this polymer release the drugs in the reducing environment of the targeted site.

2.5 Redox Environment in Wound Healing

Redox signalling is important factor for wound healing as it influences the extracellular matrix. Disruption of redox signalling affects the balance of ROS production and scavenging (Kunkemoeller et al., 2017).

CHAPTER 3

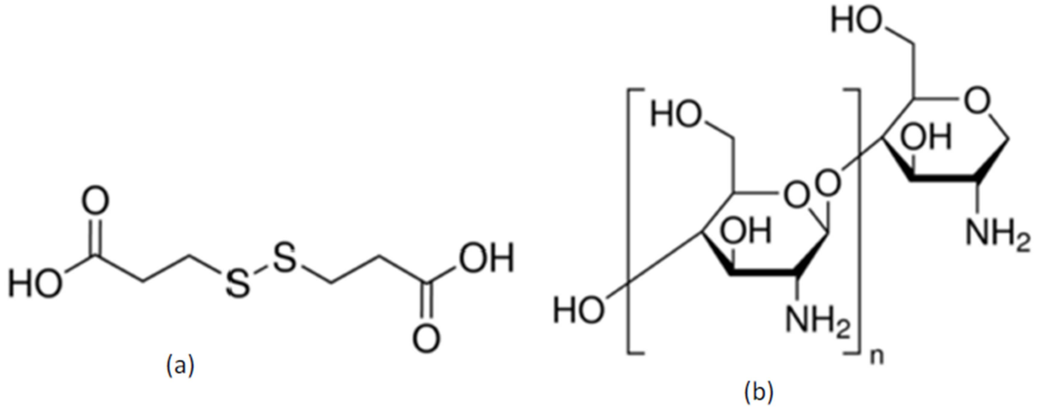
MATERIALS AND METHODS

3.1 Materials

Chitosan (low MW) purchased from SRL Pvt. Ltd., 1-Ethyl-3-[3-dimethyl amino propyl) carbodiimide hydrochloride (EDC), 3,3’-dithiodipropionic acid (DTPA), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Minimum Essential Medium (MEM), Trypsin-EDTA, Dimethyl sulphoxide (DMSO), were purchased from Sigma–Aldrich Chemicals Co, USA. Fetal bovine serum (FBS). All other chemicals were from Merck India and SRL, India and were of analytical grade.

3.2 Methods

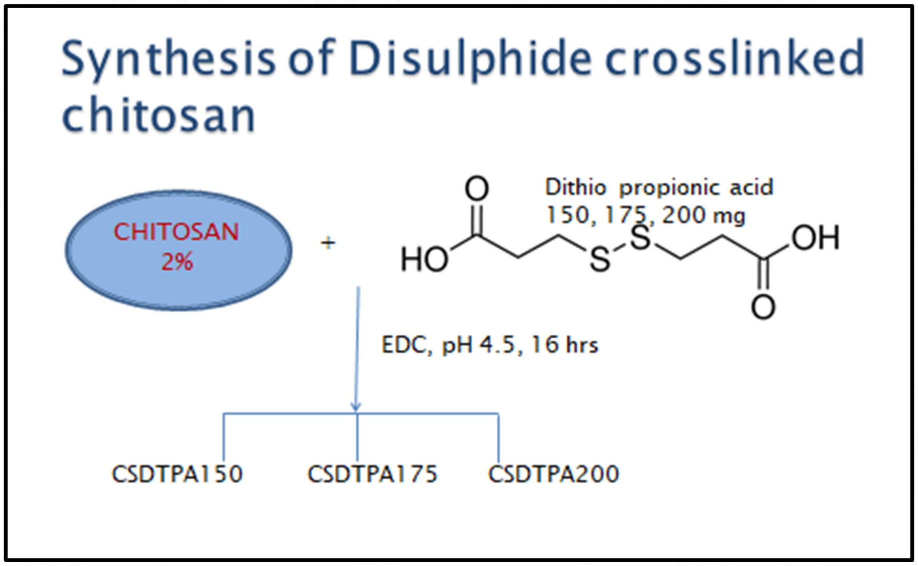
3.2.1 *Synthesis of CS-DTPA*



(a) (b)

Figure.2. (a) Dithio propionic acid, (b) Chitosan (www.sigmaaldrich.com)

Chitosan solution of concentration 2 wt% was prepared by dissolving it in 0.1M hydrochloric acid. This was stirred for 7 h until it dissolved completely and the pH was adjusted to 4.2-4.5 using 1 N NaOH. Dithio propionic acid (DTPA) of varying amounts such as 200mg, 175mg and 150 mg was dissolved in 8ml methanol to which 1g EDC was added. EDC/DTPA solutions were then added to the chitosan solution after neutralizing the pH. The conjugation reaction was done under continuous stirring. These solutions were stirred for 16 h. Acetone precipitation was done in these samples after the conjugation reaction. The precipitates are filtered using a nylon cloth and collected, then 50-60ml of water was added to these precipitates and stirred until complete dissolution and the smell of acetone disappears completely.



Scheme.1. Synthesis of CSDTPA conjugates

3.2.2. *Fabrication of CS-DTPA scaffold*

CS-DTPA samples were poured in petri plates and kept at -35°C for 24h. The frozen CS-DTPA samples were lyophilized by freeze-drying at −52°C for 48 h. Chitosan scaffolds were also prepared at 2% concentration.

3.1 Characterization of Chitosan and CS-DTPA

3.3.1 *Molecular weight determination using Ubbelohde Viscometer*

Molecular weight is one of the primary parameters in characterizing a polymer which can be determined by different techniques. Among them the simplest and fastest method is viscometry. Ubbelohde viscometer is a capillary viscometer. The viscosity is determined by measuring the time required for a definite volume of liquid to flow through capillary tube because of the hydrostatic pressure of the liquid column itself.

3.3.1.1 *Preparation of chitosan stock solution*

5mg/ml of chitosan solution was prepared by dissolving chitosan in 0.1M glacial acetic acid and stirred for 7 hours. After stirring 0.2N NaCl solution was added and overnight stirring was done.in another step 0.1M glacial acetic acid and 0.2N NaCl (1:1) was mixed to prepare the solution for diluting the chitosan stock solution in different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg/ml).the room temperature was set to 24˚C and the viscometer in level stand and sample solutions are kept in this temperature for 30 minutes. Sample solutions were filled in the viscometer between the marked levels (M_1 and M_2). Flow times of pure solvent (t_0) and different concentrations of sample solutions ($t_{0.5}$, $t_{1.0}$, $t_{1.5}$, $t_{2.0}$, and $t_{2.5}$) were determined by noting down the time taken for the solutions to reach from M_1 to M_2. The average times taken for each sample were calculated and following values were calculated using the average values;

- Relative viscosity $\eta_r = t/t_0$
- Specific viscosity $\eta_{sp} = \eta_r - 1$
- Reduced viscosity $\eta_{red} = \eta_{sp} / C$, c- concentration

- Intrinsic viscosity $Ƞ = kM^a$
- Molecular weight, $M = (Ƞ/k)^{1/a}$

3.3.2 *Determination of degree of deacetylation*

The degree of deacetylation (DD %) is defined as the molar fraction of GlcN in the copolymers (chitosan) composed of GlcNAc (N-acetylglucosamine) and GlcN (glucosamine). The DD value of a chitosan sample is one of the most important factors in assessing its applications in different fields. CS-DTPA sample solutions (200mg, 175mg, and 150 mg). A solution of concentration 0.1mg/ml were taken and dried in oven to check the dry weight. CS-DTPA solution (0.1mg/ml) diluted with 0.1 N HCL. Triplicate samples were prepared and absorbance of these samples were read at 201nm.the average value is calculated. The degree of deacetylation was calculated using the equation;

$$A/c_t = k\,DA + c$$

$$DA = \frac{(161.1 * A * V - c * m)}{(k * m - 42.1 * A * V)} \times 100$$

3.3.3 *FTIR Analysis*

For the confirmation of the modification of polymer, FTIR spectra of CSDTPA 200 and CSDTPA150 scaffolds were performed in Thermo-Nicolet 5700, FTIR spectrophotometer.

3.4. Characterisation of CS-DTPA scaffolds

3.4.1 *Porosity Analysis*

The porosity of the scaffolds was analysed using liquid displacement method. The scaffolds (CS-DTPA 200g, 175 mg, and 150mg) dry weight, W was immersed in a known volume (V_1) of ethanol in a graduated cylinder for approximately 5 minutes. The total volume of ethanol and ethanol- impregnated scaffold was recorded as V_2. The ethanol impregnated scaffold was then removed from the cylinder and residual ethanol volume was recorded as V_3.

The total volume of the scaffold, V is determined using the equation $(V_2-V_1) + (V_1-V_3) = V_2-V_3$; were V_2-V_1 is the volume of the polymer scaffold and V_1-V_3 is the volume of ethanol within the scaffold.

The porosity of the scaffold (E) is obtained by equation $E\% = (V_1-V_3/V_2-V_3) *100$

3.4.2 Tensile strength determination of CSDTPA scaffolds

The ultimate tensile strength of the chitosan and crosslinked chitosan scaffolds was measured by a universal testing machine. The scaffold was placed on the grip and was pulled to break at a constant cross head speed of 5 mm/min under 60% relative humidity until failure. A tensile stress-strain curve was generated from each sample. The average and standard deviations of measurements from three samples are reported.

3.4.3 Swelling Analysis

Swelling analysis was performed on all the scaffolds of Chitosan-DTPA of concentration 200mg, 175mg, 150mg. The scaffolds of those test specimens (mass of about 2-5 mg) were immersed in PBS (pH 7.4, 6.8, 5.4) at a constant temperature (37°C). scaffold-weight was obtained for 48 h, namely from 0 to 1 h with a step of 30 min, from 1 to 9 h with a step of 1 h, and once after 24 h and 48 h. At each time of weighing, the excess water was removed using filter paper. The swelling ratios (Q_s) of the test samples were calculated from the following equation:

Q_s (%) = $(W_t - W_0)/W_0 \times 100$, where W_t and W_0 are the weights of the swollen sample at certain time and dry test samples, respectively.

3.4.4 Determination of weight loss percentage

The weight loss percentage of the scaffolds was determined by immersing in pseudo extracellular fluid at different pH7.4, 6.8, and 5.4 for 24 h. At predetermined time-intervals (0h, 1h, 4h, 8h, and 24h); the scaffolds were removed from the medium, washed with deionized water, then subsequently frozen, and lyophilized. Weight-loss was then determined using the equation,

Weight loss % = $(W_0 - W_t)/W_0 \times 100$, where W_0 and W_t are the initial weight of the samples and final weight of the sample.

3.4.5. *Redox responsiveness of CS-DTPA*

The reduction sensitivity of CS-DTPA was evaluated by immersing the sponges in D, L-dithiothreitol (DTT) solution with a final DTT concentration of 50 mM to simulate the reducing environments. Scaffold-weight was obtained for 48 h, namely from 0 to 1 h with a step of 30 min, from 1 to 9 h with a step of 1 h, and once after 24 h and 48 h.

3.4.6. Water Vapour Transmission Rate

Lyophilized scaffolds were cut into circular pieces of diameter 18mm.Deionised water was taken in glass vials of 15ml. The cut samples were fixed in the mouth of the vial using a Teflon tape. The weight of the vials were noted down and kept in an incubator at 37˚C for a period of 6 days. The weight of the samples noted down every 24 hours. The average value is calculated and a graph is plotted as days against weight loss. The slope of straight line determined from the graph and water vapour transmission rate is calculated using the equation,

$$\text{WVTR} = \text{slope of straight line/surface area}$$

3.4.7 In vitro Drug Release study

The drug release study was done by placing the CSDTPA sponges (200mg, 175mg, and 150mg) in a 50 ml beaker. The sponges are loaded with 100µl of insulin, to this 5ml of PBS (pH6.8) was added. At each predefined time interval (0-8h) 200µl of release medium was withdrawn and replaced with an equal volume of corresponding fresh media. The release percentage was determined by Lowry's method.

3.5 Cell Culture

L929 mouse fibroblast cells were cultured in MEM medium. All cells are cultured in a humidified incubator at 37˚C with 5% CO_2 atmosphere supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (streptomycin and penicillin).

3.5.1. *In vitro Cytotoxicity-MTT Assay*

The cytotoxicity study of CSDTPA (200mg, 175mg, and 150mg) were done by seeding the cells in 96-well plates at a density of cells/well and cultured overnight to allow cells to get attached. The medium was then replaced with refresh medium and make stock having 100µl of sample (CS-DTPA 200mg, 175mg and 150mg) made up to 1ml using FBS. From these, 12.5, 25, 37.5 and 50µl were taken and added to the cell layer and made up to 200µl using media.

After incubation at 37˚C, 5% CO_2 for 24h, samples were removed and MTT reagent (0.5mg/ml) was added to each well. Again incubated for 3hours then the reagent was removed and dimethyl sulfoxide (DMSO) added to dissolve the MTT formazan crystals then it is incubated for 20 minutes. The cell viability was calculated as percentage relative to control cells. The absorbance was readed at 570nm. MEM with 10% FBS is taken as negative control and triton-x 100 as positive control.

The toxicity of CS-DTPA scaffold was evaluated using test on extract method. Scaffold samples of 10mg size was extracted with 5ml of phosphate buffer pH 6.8 for 24 hours and different volume of 12.5, 25, 37.5 and 50µl were taken and added to the cells and made up to 200µl using media.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of Chitosan and CS-DTPA

The characterisation of chitosan was done by carrying out molecular weight determination by Ubbelohde method and analysing degree of deacetylation.

4.1.1 *Molecular weight determination of chitosan using Ubbelohde Viscometer*

The reduced viscosity and inherent viscosity of chitosan was calculated and obtained (figure.3). The intrinsic viscosity $[\eta]$ was calculated graphically by extrapolating the curve of reduced viscosity versus concentration to zero concentration. The molecular weight was then calculated by using Mark-Houwink equation:

Molecular weight, M = $(\eta/k)^{1/a}$ were $\eta = 2.4$, $k = 1.81*10^{-5}$, a= 0.93

$$= (2.4/1.8*10^{-5})^{1/0.93}$$

$$= 322 \text{ kDa}$$

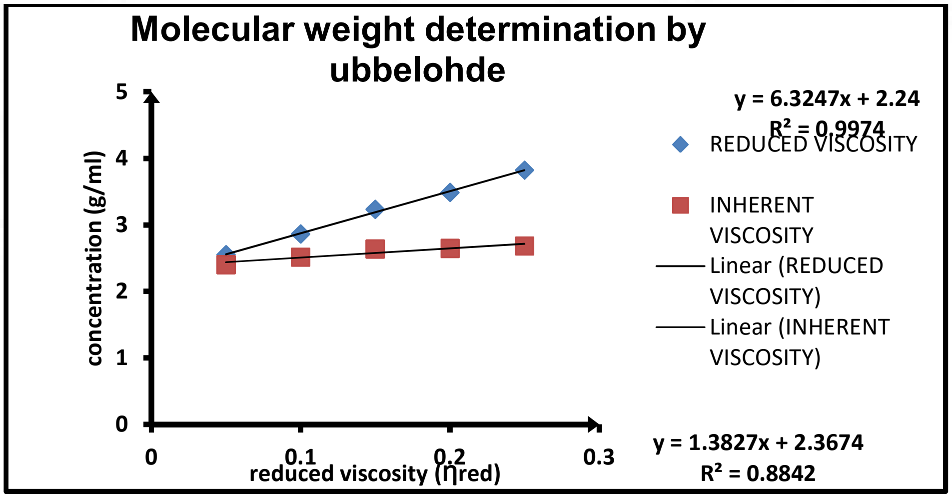


Figure.3.Molecular weight determination by Ubbelohde viscometer

4.1.2 *Determination of degree of deacetylation*

The determination of degree of deacetylation was determined by UV-spectrophotometric analysis using dual standards of N-acetyl glucosamine and glucosamine. The calibration curve is shown figure.2. Degrees of deacetylation quantification result showed that chitosan have DD of 72.98 %. On the other hand, Degree of deacetylation values differed when chitosan is conjugated with DTPA. Degree of deacetylation values of Chitosan- DTPA conjugates of different concentrations (150 mg, 175 mg and 200 mg) is estimated by applying test absorbance values(A150 = 0.6002, A175 = 0.0696, A200 = 0.5457) in the equation ;

DA = $\frac{(161.1*A*V-c*m)}{(k*m-42.1*A*V)}$ x 100 and it is turned to have 65.63 %, 65 % and 69 % respectively.

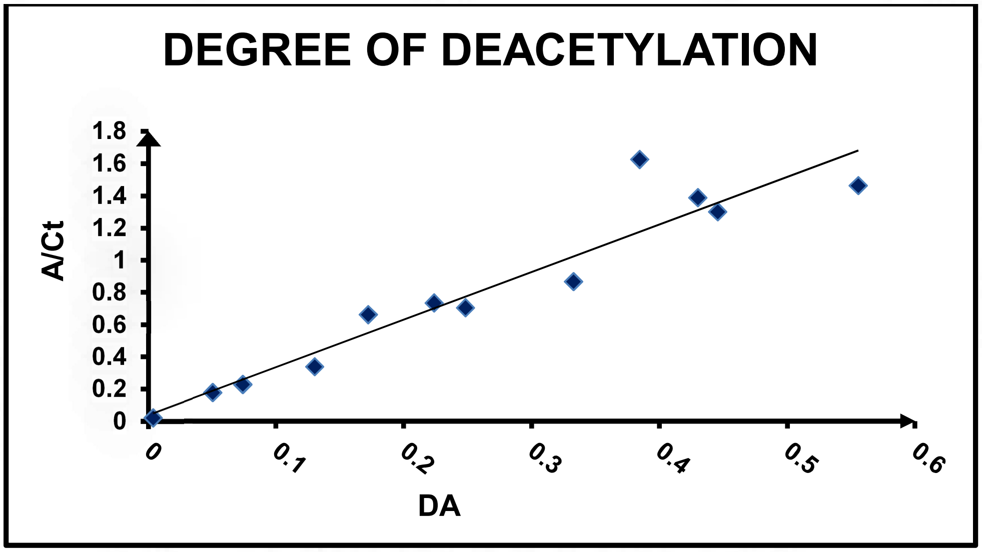


Figure.4.Degree of deacetylation

Molecular weight and Degree of deacetylation are the crucial parameters and that has pronounced influence on the stability, mechanical strength and degradation properties of chitosan and its derivatives.

4.1.3 *Synthesis of CS-DTPA and FTIR Analysis*

Disulphide cross linked chitosan was developed by conjugating chitosan with dithio propionic acid using EDC as the cross-linker. To a fixed quantity of chitosan varying amount of DTPA was added, viz., 150, 175 and 200 mg respectively. The obtained products were coded as CSDTPA150, CSDTPA175 and CSDTPA200.

To evaluate the conjugation of dithio propionic acid to chitosan FTIR analysis was carried out. As shown in figure.5.a FTIR spectra of CSDTPA200, shows the bands detected at 899 cm^{-1} assigned to C-O-C bonds. The two peaks at 1018 and 1065 cm^{-1} were assigned to the C-N bond, and the two peaks at around 1650 and 1550 cm^{-1} correspond to C=O and N-H bonds, respectively. The peak at 3265 cm^{-1} shows a medium N-H stretching. The peak at 2919 cm^{-1} shows a medium C-H stretching.

In figure.5.b shows the FTIR spectra of CSDTPA175, the bands detected at 899 and 654 cm^{-1} are assigned to C-O-C bonds. The two peaks at 1019 and 1065 cm^{-1} were assigned to the C-N bond, and the two peaks at around 1650 and 1550 cm^{-1} correspond to C=O and N-H bonds, respectively. The peak at 3309 cm^{-1} shows a medium N-H stretching. The peak at 2895 cm^{-1} shows a medium C-H stretching.

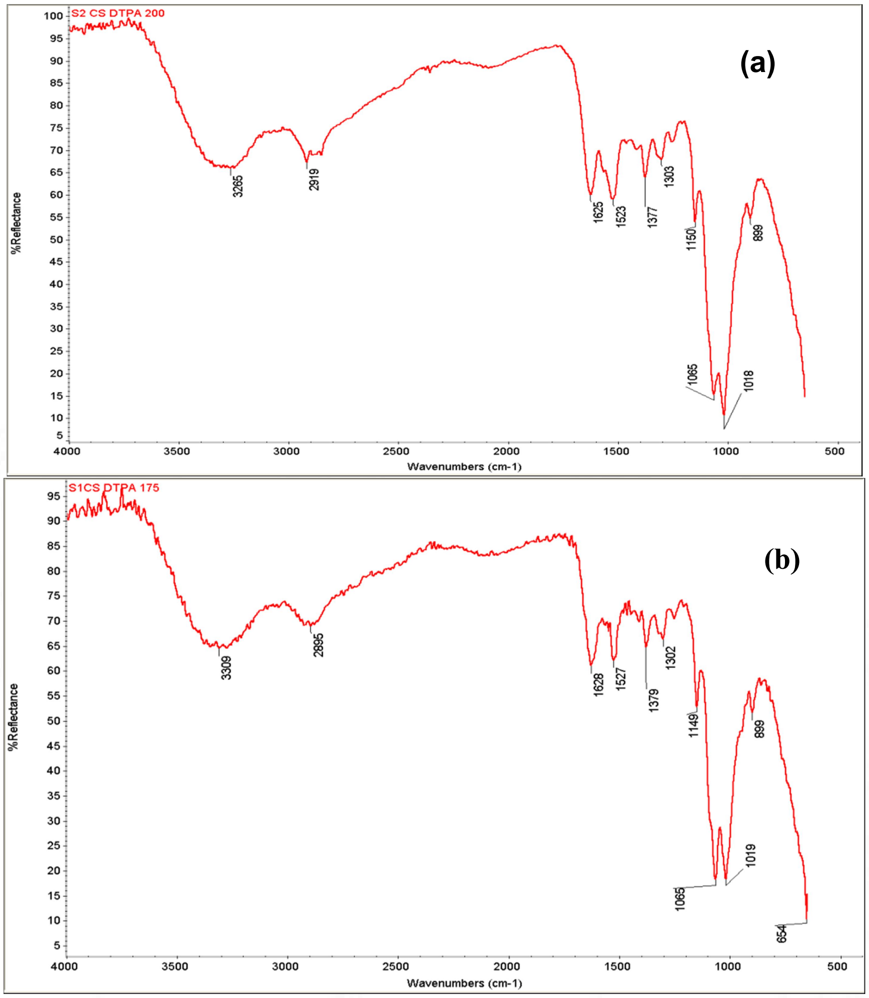


Figure.5.FTIR spectra of CS-DTPA conjugates, (a) represents 200mg and (b) 175 mg conjugate

4.2 Characterisation of CS-DTPA scaffolds

Scaffolds were developed from DTPA conjugates by freeze drying method. The scaffolds were then evaluated as discussed in the coming sections.

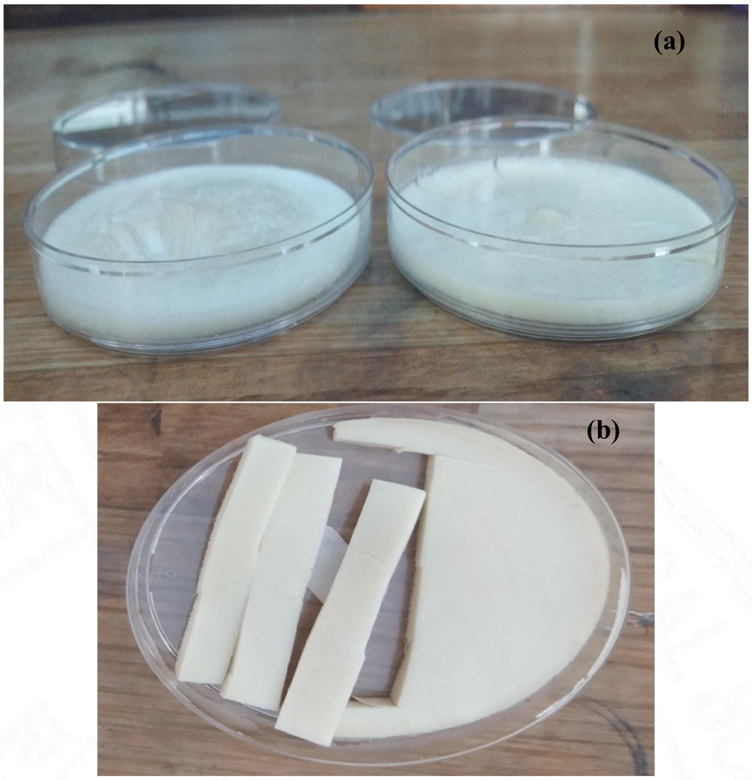


Figure.6. Images of CSDTPA conjugates

4.2.1 *Porosity Analysis*

The porosity of chitosan (2 wt %) scaffolds developed with different concentrations of DTPA (200 mg, 175 mg, and 150 mg) is shown in figure 4. By comparing the porosity of the three CS-DTPA scaffolds we can see that, CS-DTPA of concentration 200 mg have 50 % porosity. CS-DTPA of concentration 175 mg shows porosity of 72.2%. CS-DTPA of concentration 150 mg shows porosity 86.6%. As the crosslinking of the scaffolds increases the porosity percentage of the scaffold is decreases.

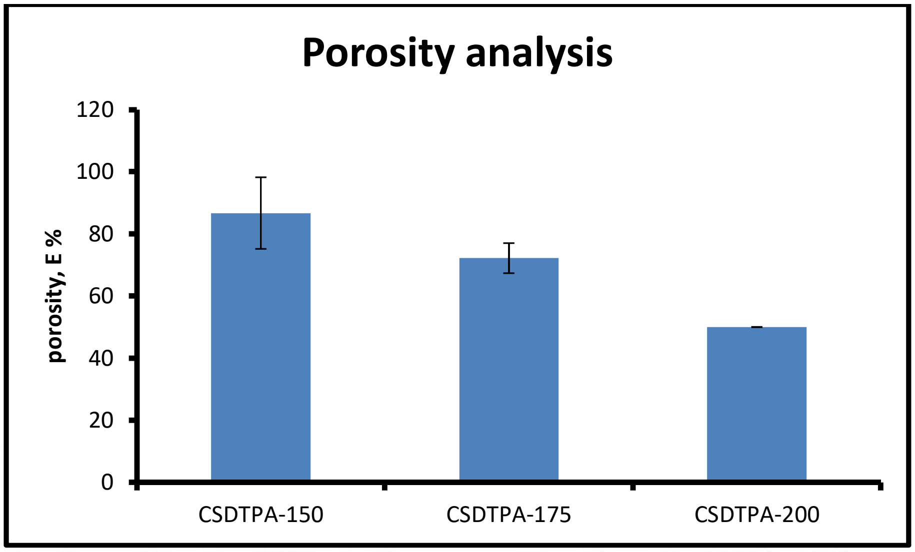


Figure.7. Porosity analysis

4.2.2 *Tensile strength determination of CSDTPA Scaffolds*

The figure.7 shows the tensile strength analysis of the chitosan and CSDTPA scaffolds. The tensile strength of crosslinked scaffolds (CSDTPA200, CSDTPA150) were analysed using tensile strength measurements. The tensile strength values were 1.35 ±0.00 and 0.32 ± 0.09 MPa for CSDTPA200 and CSDTPA150 respectively. The tensile strength of the chitosan scaffold used as a control was 0.50 ± 0.01MPa. The porosity also influences the mechanical properties of scaffolds (Takeshi et al., 2014). So here, as the porosity of the scaffold increases the tensile strength of the scaffold decreases. Here the results show that crosslinked CSDTPA200 scaffold has higher tensile strength compared to the Chitosan scaffold and CSDTPA150 scaffold shows low tensile strength.

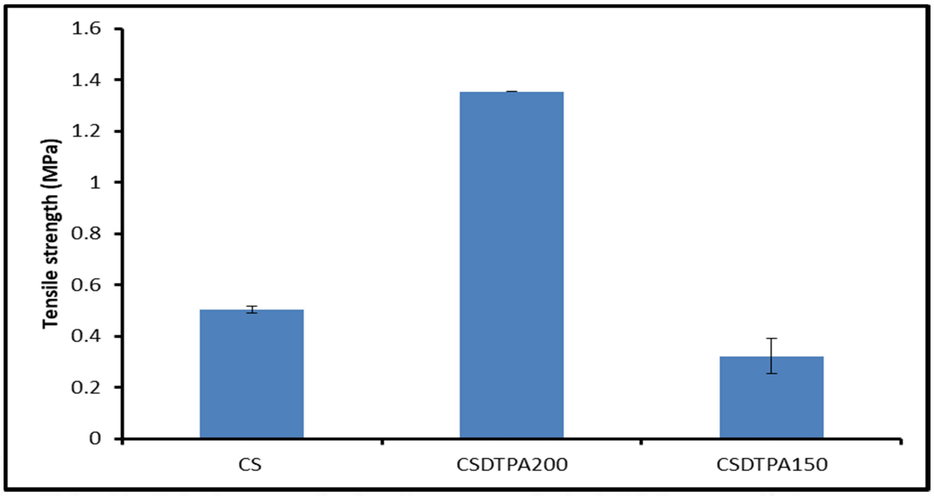


Figure.8.Tensile strength analysis of Chitosan and CSDTPA Scaffolds

4.2.3 *Swelling Analysis*

The figure.8.a shows the percentage swelling of CS-DTPA conjugate of concentration 200 mg in different pH (7.4, 6.8, and 5.4). CSDTPA200 in pH7.4 shows a swelling percentage range of 1200-1400. At 7 hr the swelling decreased then at 8hr the swelling increased and became stable. In pH6.8 the swelling percentage shows a range of 1100-1700. The swelling percentage increased in 4th hr. in 9th hr and 24th hr the swelling of the CSDTPA200 is decreased. In pH5.4 the scaffold shows a swelling range of 2700-3100.

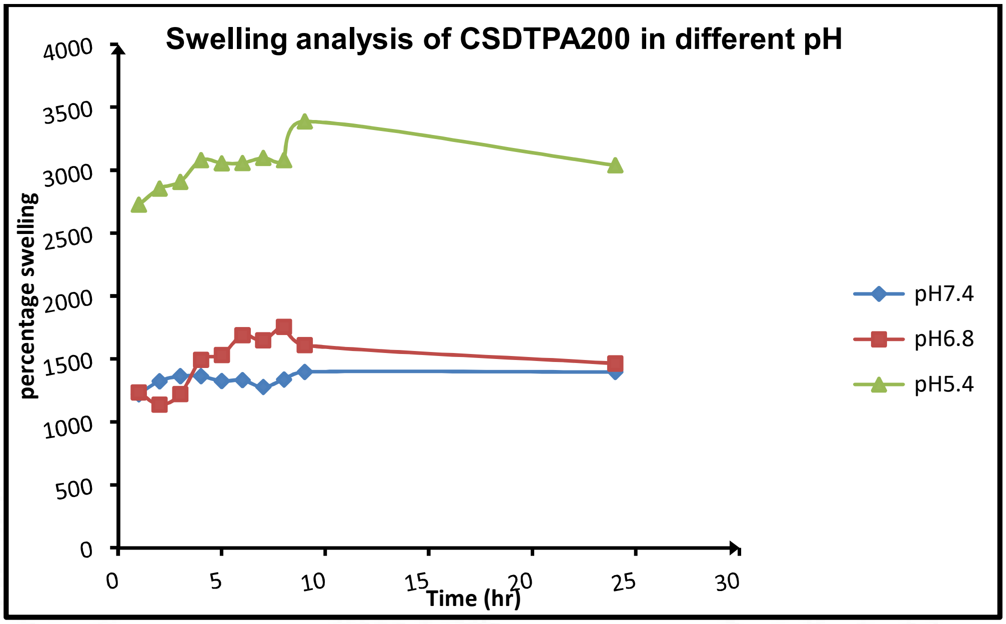


Figure.9.a Percentage swelling of CSDTPA200 in different pH

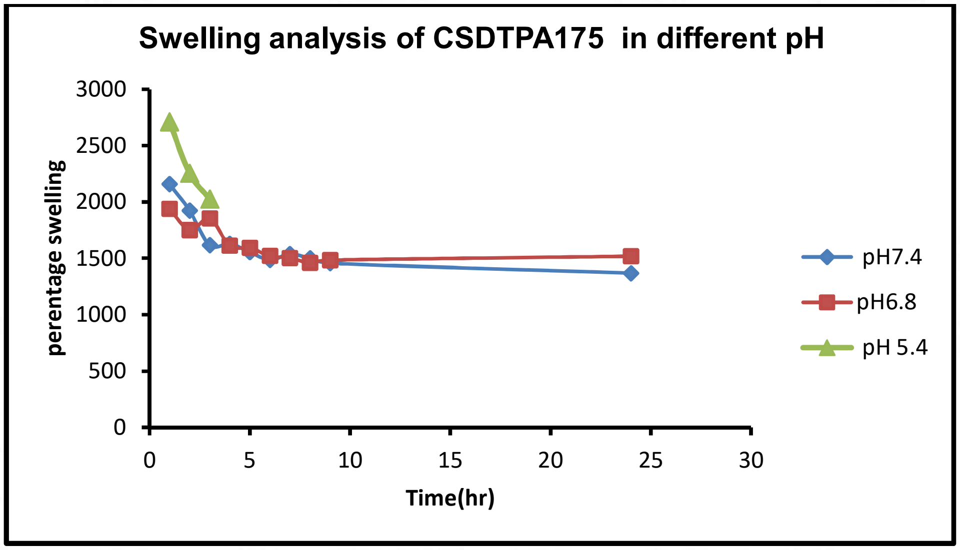


Figure.9.b. Percentage swelling of CSDTPA175 in different pH

From the figure.8.b, it can be depicted that swelling behaviour of CSDTPA175 start to drop after 1 hr in pH6.8 and 7.4 ranges. Even though, there is a modest increase in swelling at pH 6.8, it is again lowered after 3rd hr. in addition degree of swelling becomes invariable in pH 6.8 and pH 7.4 after 9th hr. in case of pH5.4 percentage swelling decreased after 3rd hr and dissolved in the buffer of pH5.4.

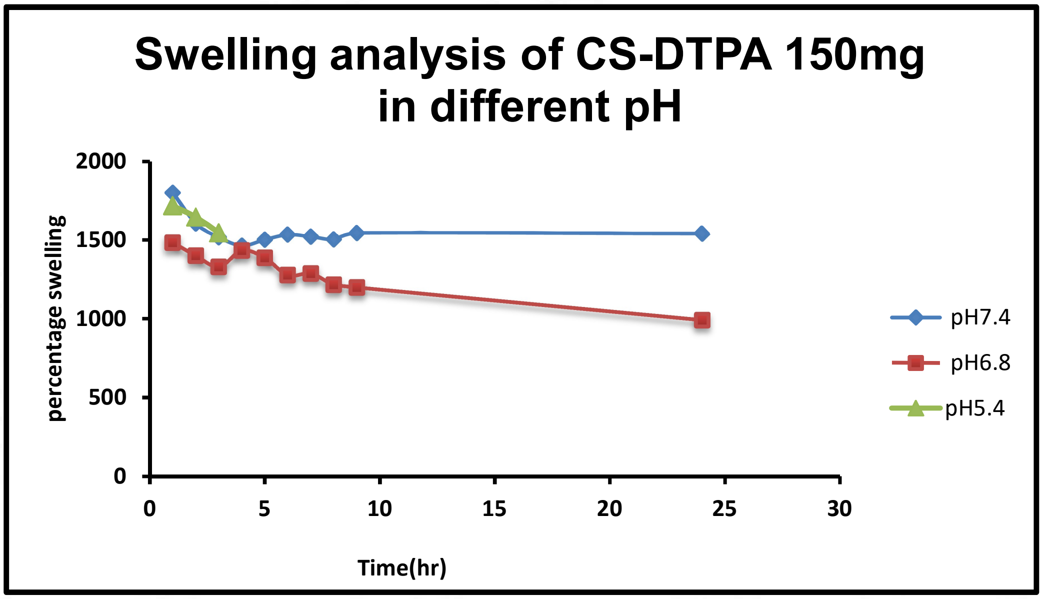


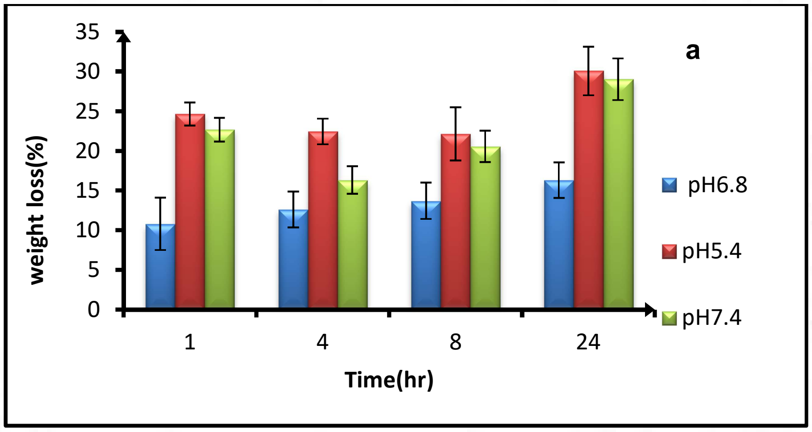
Figure.9.c. Percentage swelling of CSDTPA150 in different pH

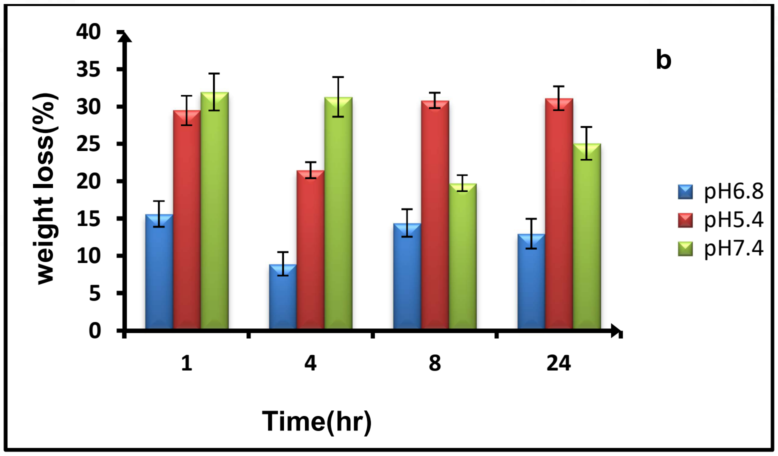
From these graphs we can interpret that CSDTPA200 scaffold is stable in all pH for 24 hr. It has good swelling capacity as it will be used for drug delivery. The scaffold CSDTPA175 and CSDTPA150 is stable in pH6.8 and 7.4 and unstable in pH5.4. As the scaffold CSDTPA200 showed stability in all pH, in the 24hr time period and the degradability is lower, it is suitable to use for drug delivery.

4.2.4 *Determination of weight loss percentage*

In figure.9.a show the dissolution of CSDTPA200 in different pH. As shown in the graph it can be seen that CSDTPA200 shows lower weight loss percentage rate at pH5.4 at 1 hr and it gradually increases by 24th hr. At pH 6.8 and 7.4 the dissolution is higher even at 1 hr and increases to around 30% by 24 hours.

The CSDTPA175 has shown higher dissolution in pH5.4 and while looking into the swelling percentage of the conjugate in previous data (figure.5.b) after first 3 hr the conjugate dissolved in pH5.4 because of the solubility of lesser conjugated chitosan in acidic pH.





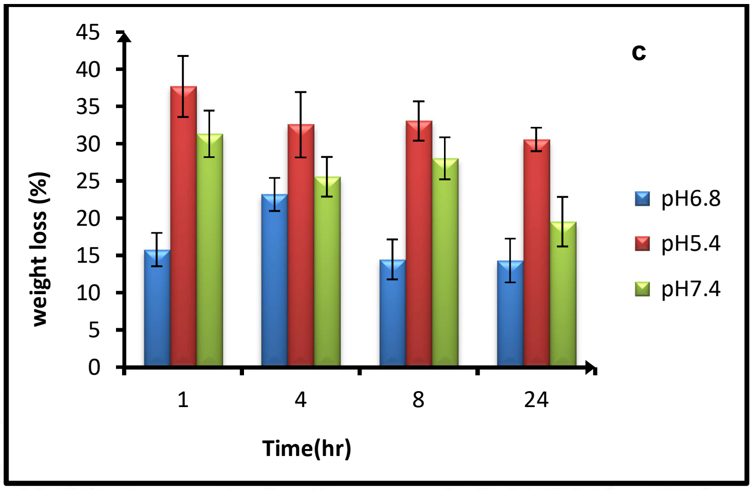


Figure.10. weight loss percentage rate of (a) CSDTPA200, (b) CSDTPA175 and (c) CSDTPA150 in different pH

In figure.9.c has shown the dissolution analysis of CSDTPA150 in different pH. The scaffold in pH6.8 shows a decreased weight loss percentage rate; in 4th hr the weight loss % rate is higher but decreased in 8th and 24th hr. in pH5.4 at 1st hr the weight loss % rate is higher and after 1 hr the weight loss % rate has decreased. In pH7.4 also shown an increase in weight loss % rate at 1st hr then the scaffold has shown a decreased weight loss % rate at 24th hr.

In pH5.4 weight loss % rate of conjugate gets decreased at 24th hr comparing with the previous data of swelling percentage (figure.8.c) which was decreased as the conjugate got dissolved in pH5.4 in swelling analysis.

4.2.5 *Redox responsiveness of CS-DTPA*

Redox responsiveness of CSDTPA conjugates having DTPA concentration 200mg, 175mg and 150mg were analysed by immersing the conjugates in DTT(50mM) loaded PBS buffer of different pH

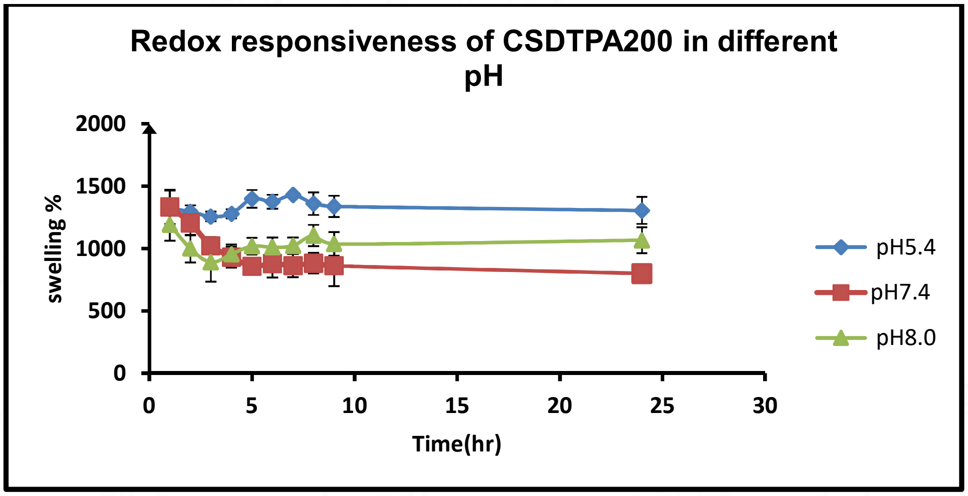


Figure.11.a. Redox responsiveness of CS-DTPA in different pH

In figure.10.a shows the redox responsiveness of CSDTPA200 in different pH. In pH5.4 the scaffold swelling percentage ranges between 1430-1200. The scaffold shown higher swelling in 7th hr but the swelling percentage decreased after 7th hr.in pH7.4 the swelling percentage decreases after 1 hr and stays in the range of 1300-800. In pH8.0 the swelling percentage decreased after 1 hr then showed a moderate increase in 5- 9 hrs. In 24th hr the swelling percentage was decreased.

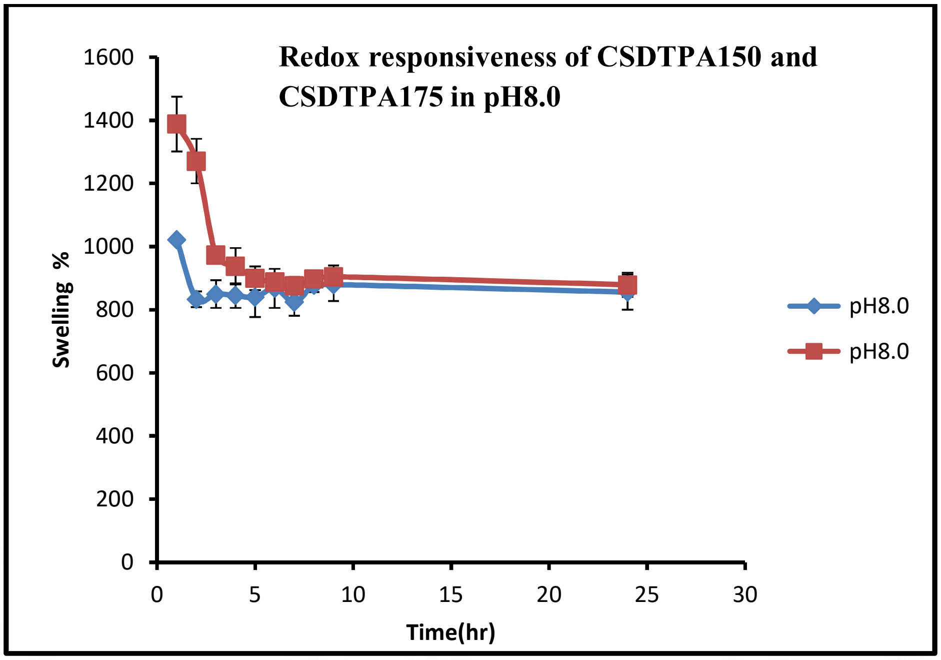


Figure.11.b. Redox responsiveness of CSDTPA150 and CSDTPA175 in pH8.0

The figure 10.b. shows the redox responsiveness of CS-DTPA 150mg and CSDTPA175 in pH8.0. In pH8.0 the CSDTPA150 scaffold shows a swelling percentage of 1000-800. The swelling percentage shows slight variation decreases after 1 hr and stays in the range of 800. The CSDTPA175 shows a swelling percentage range of 1300-900. The scaffolds swelling percentage has shown a decrease in pH8.0.

4.2.6 *Water Vapour Transmission Rate (WVTR)*

The ability of scaffolds to reduce body liquid loss is an important factor for healing the wound. Therefore, WVTR was analysed for the scaffolds CSDTPA200, CSDTPA175 and CSDTPA150. WVTR was calculated using the equation;

WVTR ($g/m^2/day$) = Slope of the straight line/Surface area

Diameter of sample = 18mm

Surface area = $\pi r^2 = 0.00025434\ m^2$

WVTR of CSDTAP200 = 0.3789 /0.00025434 = 1489.78 $g/m^2/day$

WVTR of CSDTPA175 = 0.3846/0.00025434 = 1512.15 $g/m^2/day$

WVTR of CSDTPA150 = 0.5206 /0.02826 = 2046.87 $g/m^2/day$

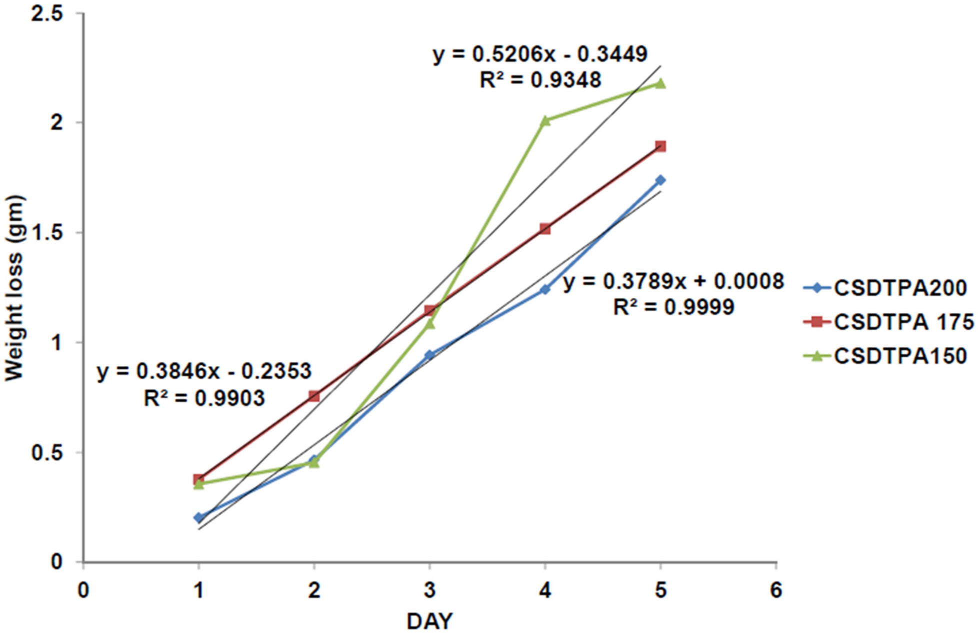


Figure.12. Water vapour transmission rate analysis of CSDTPA scaffolds

The main factor for wound healing scaffold is an optimal WVTR. An ideal scaffold for wound healing should be able to control the evaporative water loss from a wound surface. WVTR for normal skin is 204 g/m^2 per day, for injured skin, it can range from 279 g/m^2 per day to 5,138 g/m^2 per day. The opted rate is 2,500 g/m^2 per day, the mid-range of loss rates, can provide a sufficient level of moisture and wouldn't risk wound dehydration (Ujang et al., 2014). If the WVTR for a scaffold is less than that of normal skin, tissue will dehydrate, and the exudate between wound and the covering can cause infection. Therefore, materials for wound dressing should have a higher WVTR value than normal skin. Here it can be seen that as crosslinking increases the WVTR decreases. The WVTR for the scaffolds decreased in the order CSDTPA200<CSDTPA175<CSDTPA150. However the water vapour transmission rates were in the ideal range suitable of wound healing material so as to maintain a moist environment.

4.2.7 *Insulin release study*

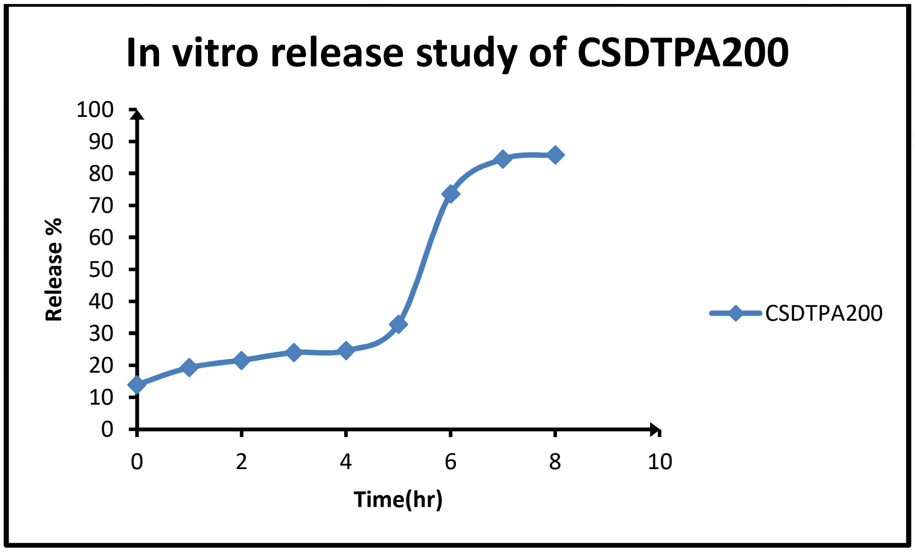


Figure.13. *In vitro* release study of CSDTPA200

In figure.9 shows the release percentage of insulin loaded (100µl) CSDTPA200 conjugate. The release percentage is gradually increasing and shows a release percentage within 100%. The drug shows a moderate increase in release percentage in 6hrs.In 8hrs CSDTPA200 shows 85% release. The CSDTPA200 shows decreased swelling behaviour and the CSDTPA200 shows no corresponding release. This needs further study to find out the reason.

4.3 *In vitro cytotoxicity and MTT-Assay*

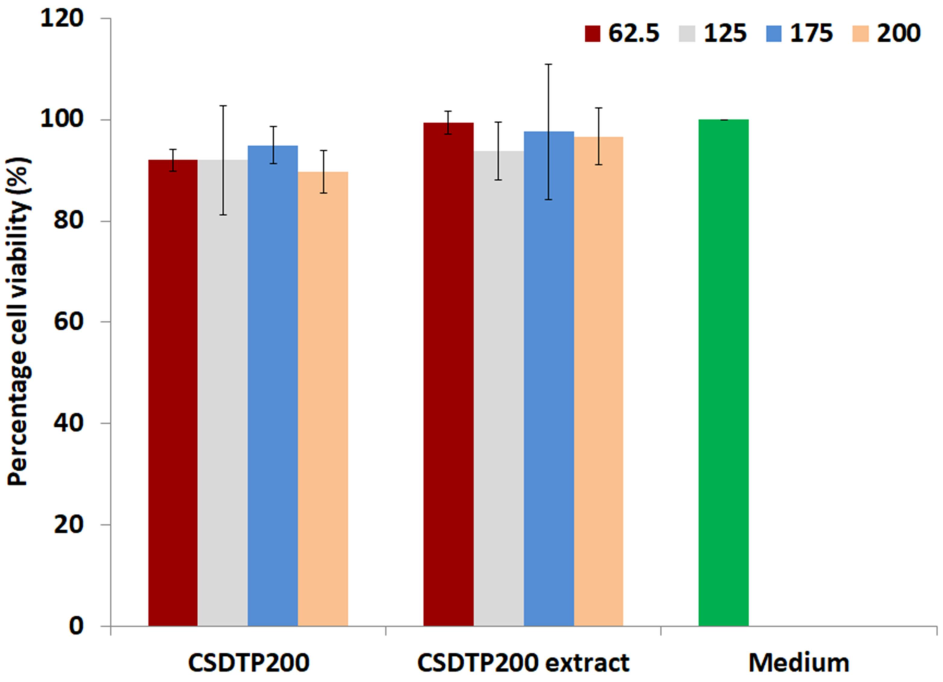


Figure.14.Percentage cell viability following exposure to CSDTPA200 and extract from CSDTPA200 scaffold at a concentration of 62.5, 125, 175 and 200 microgram/ml

In figure.10 cell viability percentage of both CSDTPA200 and CSDTPA200 extract was shown. Both the chitosan dithio propionic acid derivative (CSDTPA) and its scaffold were found to be highly cytocompatible with more than 90% cell viability.

CHAPTER 5

SUMMARY AND CONCLUSION

In the present study dithio propionic acid crosslinked chitosan (CSDTPA) based scaffolds were developed and evaluated its efficacy as a wound care biomaterial. Physico-chemical characterization of the CSDTPA conjugates was done. In characterization of chitosan and CSDTPA conjugates molecular weight of chitosan was determined using Ubbelohde viscometer and Degree of deacetylation of CSDTPA conjugates was also analysed using UV spectrophotometric methods using dual standards. Molecular weight and degree of deacetylation are crucial parameters which influence the stability, mechanical strength and degradation properties of the product. For confirming the conjugation of DTPA with chitosan FTIR analysis was done. Physico chemical characterisations of the scaffolds were done by analysing the porosity, tensile strength, swelling ratio, water vapour transmission rate, weight loss percentage of the scaffolds. Being scaffolds bearing disulphide linkages the swelling studies were carried out in presence of DTT as well. Swelling behaviour of the CSDTPA conjugates were studied in different pH. The CSDTPA200 conjugate showed stability in all pH. The CSDTPA175 and CSDTPA150 scaffolds were unstable in acidic pH. In weight loss percentage analysis CSDTPA200 shows increased rate. CSDTPA175 and CSDTPA150 showed decreased weight loss percentage. The redox responsiveness of the conjugates was also studied using DTT, a reducing agent. In 24th hr the CSDTPA200 conjugate showed decreased swelling in all pH. CSDTPA175 and CSDTPA150 shown decrease in swelling after a time period. Water vapour transmission rate was checked for the three conjugates for analysing the ability of scaffolds to reduce body liquid loss. The drug releasing ability of the scaffold was also checked and the CSDTPA200 scaffold shown release percentage within the standard curve. But the CSDTPA200 scaffold shows no correlation with swelling percentage. This needs further analysis. In tensile strength analysis cross linked CSDTPA200 scaffold was found to have higher tensile strength compared to chitosan. In cytotoxicity - MTT assay the CSDTPA200 and CSDTPA200 extract were shown above 90% cell viability. As of now CSDTPA200 scaffold was chosen to be the one to be further analysis as it shows stability in all pH.

5.1 FUTURE PERSPECTIVES OF THE STUDY

1. Effect of released drug on different cell lines (fibroblast and keratinocytes).
2. *In vitro* scratch wound assay using cell lines.
3. To extend the study to in vivo animal models to analyse the ability of the conjugates with disulphide linkage in therapeutic molecule delivery.

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