Chapter 5- Summary, conclusion and future prospect

Skin tissue engineering has emerged as an alternative promising tool to repairing and/or replacing damaged and/or diseased skin tissue. In tissue engineering, design and developmet of scaffold with desired properties such as biocompatibility, biodegradability, suitable mechanical strength with tissue regenerative potential are the principle challenges before the researcher for developing the skin tissue construct. Collagen, most abundant extracellular matrix protein present in skin is suitable for tissue construct for skin, but collagen has some inherent disadvantage associated with it such as poor mechanical strength. Therefore it is mixed with other natural polymer such as chitosan, which has good mechanical properties to provide the desired mechanical strength to the construct. Infection in wound is very serious issue which lead to the sepsis and delay in wound healing. Therefore antimicrobial agents as antibiotics are generally used to control the infection at wound site. Hypoxia is another critical issue related to wound healing. Since wound healing is an active phenomenon, it required high amount of nutrition and oxygen. Due to lack of the vascularisation in wound site the presence of oxygen become the limiting factor during wound healing, therefore oxygen generating material are encapsulated inside the scaffold which provide the oxygen to avoid the hypoxia and accelerates wound healing.

The successful scaffold-based skin tissue regenerative approach dependents on many factors which includes the selection of biomaterial used in scaffold preparation, fabrication technique, use of strategies to prevent infection and hypoxia during wound healing.

The most encouraging observations obtained from this research work are summarised below:

In the scaffold material based study, different combination of collagen and chitosan were used to obtain the scaffold using freeze drying technique. Freeze drying technique is used

because it provide the high porosity with pore size which is suitable for the growth, migration and proliferation of fibroblast. Dual cross linking was found more effective than the single cross linking. As dual cross linking with EDC/NHS and glutaldehyde help to maintain the architecture of scaffold, by maintaining the structure and morphology of pore and its connectiveity with surroundings.

Structural and physical properties of developed ciprofloxacin loaded collagen-chitosan scaffold were studies using various parameter as degradation pattern, swelling, water vapour, pore size and porosity, transmission rate, Mechanical properties etc. Various techniques as FTIR, XRD, SEM were used to conclude the physical morphology of scaffold and chemical constituents of scaffold and their crystallinity.

Physical and morphological characterization of scaffold shows the interconnected micro and macro pores. Pore size of scaffold varies from the 49μm-169 μm with average pore size of SC-1, SC-2 and SC-3 is 71,125 and 142 μm respectively. Porosity of developed scaffold was more than 90% which is desirable for nutrient and oxygen transfer. Swelling and biodegradation is an important parameter taken in consideration for scaffold. The SC-2 has shown the optimum swelling which does not negative effect the growth and migration in one hand while maintain the structural integrity. SC-3 shows maximum biodegradation whereas SC-1 shows least biodegradation while SC-2 show optimum degradation which may promotes wound healing. FTIR study confirms the presence of characteristic group of collagen, chitosan, ciprofloxacin and their interaction during cross linking process. Tensile strength of developed scaffolds was measured in dry and wet condition. Tensile strength of SC-2 was found greater compare to SC-1 and SC-3. Tensile strength of SC-1, SC-2 and SC-3 was measured 2.8, 7.2, 6.7 MPa in dry condition and 1.58, 2.62 and 2.40 MPa in wet

condition respectively. Thermal properties of developed scaffolds indicate that scaffolds are stable and maintain the structural properties intact during the initial phase of heating. Drug release study of scaffold suggest rapid and high release of antibiotics (78-93%) due to highly porous structure of scaffold. Hemocompatibility studies demonstrated that the entire developed scaffolds are highly hemocompatible.

Fibroblast were isolated from the rat skin using trypsin and collagenase enzyme. Isolated cells were cultured in CO_2 incubator at 5% CO_2 and 95% relative humidity at 37^0C using cell culture medium which contains the DMEM-L glucose, supplemented with 10% FBS, 0.1% antibiotic-antimycotic solution in T-25 cell culture flask until the 70–80% confluency was attained. Sterile scaffold were taken for cell culture studies and fibroblast were seeded 10^5 cells per scaffold for predetermined period of time. Scaffolds were washed after different time intervals and various studies such as morphological studies of fibroblast seeded scaffold were performed using SEM and fluorescence microscopy. SEM images of scaffolds shows that in SC-2 growth, migration and proliferation of fibroblast occur better than rest of scaffold because SC-2 retains its mechanical and physical integrity during the course of cell culture whereas SC-1 & SC-3 fail to support the growth and migration of fibroblast over the period of culture period. Cell proliferation was quantified through DNA quantification which further suggested that SC-2 promotes the proliferation of the fibroblast over the other scaffolds. Similar result was also concluded through MTT assay which suggest that due to suitable pore size, mechanical integrity and optimum swelling of SC-2 metabolic activities is higher in compared to SC-1 and SC-3. Based on the above morphological, physical, biochemical and cell culture analysis SC-2 was selected as best scaffold for antimicrobial &*in vivo* studies. Antibacterial study of selected scaffold (SC-2) were performed in LB

media against the *E. coli* (Gram negative) and *S. aureus* (Gram positive) which are most common occurring bacteria affecting the wound. Scaffold show clear zone of inhibition against both type of bacterial hence it contains the antibacterial properties due to ciprofloxacin antibiotic.

In vivo study was performed using skin flap model in rat model conducted according to the institute guideline. Wound in rat was created after anesthetizing them using ketamine and xylazine and full thickness wound , with dimension of 1.5 cm×1.5 cm was created. Rats were divided in two groups. First group is control, which is devoid of scaffold and second is scaffold treated test group. Wound healing is quantified by taking picture of healed wound at different time and analyzed using Image J software. In vivo result indicated that scaffold positively affects the wound healing rate and accelerated the process of healing. This may be due to prevention of infection in wound site. Another factor which may promote the wound healing may be the degradation of scaffold, scaffold upon degradation used by wound and help in accelerated wound healing. Histological studies of wound healing were performed using Hematoxylin–Eosin staining after 15 day of wound healing. Histological studies revels that process of epidermis and dermis formation and remodelling of ECM were lower in control compare to test.

Hypoxia and infection is key concern for modern regenerative medicine. Hypoxia leads to the production of reactive oxygen species and infection leads to sepsis that delays wound healing. Therefore oxygen releasing molecules such as calcium peroxide, magnesium peroxide, hydrogen peroxides are used in biomaterials to produce oxygen, that prevent hypoxia during wound healing. Oxygen releasing scaffold is fabricated using 1.5 % collagen and 1.5 % chitosan in 1:1 ratio. Ciprofloxacin was added in the solution so that the final

concentration of antibiotics was 1mg/ml. collagen chitosan solution was freeze dried using lyophilizer. Scaffolds were dual cross linked using EDC/NHS and glutaraldehyde. After cross linking scaffolds were coated with CPO and PCL. Depending on the coating of CPO of 1%, 2%, 3% and 4% the scaffolds are denoted as SC-1, SC-2,SC-3 and SC-4 respectively. SC-0 was devoid of PCL and CPO coating.

The surface morphology of developed scaffolds was studied using SEM and it was observed that pores were uniformly distributed and inter connected. CPO deposition were present over the surface of the scaffolds except SC-0. Deposition of CPO is in following order SC-4>SC-3>SC-2>SC-1>SC-0. Elemental analysis using EDX also suggest the same. Porosity of scaffolds decrease as the percentage of CPO coating increases. highest porosity was for SC-0 and least was for the SC-4. The SC-0, SC- 1, SC-2, SC-3, and SC-4 have porosity 95.2 ± 1.2, 93.3 ± 0.4, 91 ± 0.5, 89.6 ± 0.7 and 87.42 ± 0.4 respectively. Swelling is an important parameter need to consider during the during scaffold design. SC-0 highest swelling. SC-0 is devoid of PCL and CPO therefore it does not have hydrophobic coat on the surface that will lead to higher swelling. Scaffolds from SC-1 to SC-4 have gradual increase in CPO and PCL that leads to gradual decrease in swelling rate. Biodegradation is another essential hallmark and rate of scaffold degradation should meet the rate of tissue formation is analyzed using lysozyme. It is observed that SC- 0 has maximum rate of degradation rate compare to other. Other scaffolds from SC-1 to SC-4 has gradual increases in CPO that has led to accumulation of PCL in the scaffold; therefore, a lower rate of degradation has been observed from SC-4 to SC-1. FTIR and XRD analysis is used to study functional and chemical character of scaffolds. FTIR shows the characteristic peaks of collagen, chitosan, ciprofloxacin, calcium peroxide and PCL. XRD analysis indicated prominent peak at around 2θ of $21^0$and 36^0which

can be attributed to the PCL and CPO has a characteristic peak at 41.31^0. Collagen-chitosan complex has distinct peaks associated 24.7^0, 27^0, and 30.90^0. The XRD peak intensity defines the quantity of constituent material. As the constituent material increases as the intensity of XRD peaks associated also increases. Present study attempt to design and evaluate the oxygen releasing scaffold to control the hypoxic condition. When oxygen release was measured and it was found dependent on the amount of CPO coating at the scaffold. No sudden release of oxygen was observed. Since SC-0 is devoid of CPO no release of oxygen was observed. Scaffold is loaded with antibiotic to provide the protection against the microorganism. Antibiotic release depends upon the various factors which included, solubility, permeability and crystallinity of drug, structure of scaffold, its porosity, degree of cross-linking, incorporation of coating with polymer and the nature of the polymer used in the coating etc.

Since all scaffolds are similar in nature, they differ only in the concentration of CPO and the amount of PCL in the scaffold. Higher CPO content lead to higher PCL associated with it, which lead to higher hydrophobicity associated with scaffold. Higher hydrophobicity in scaffold lead to prolonged, sustained and continuous release of ciprofloxacin. SC-0 have no PCL coating therefore it show sudden release of ciprofloxacin while PCL and CPO content associated with scaffold has successively increased form SC-1, SC-2, SC-3 and SC-4 from 1% to 4% that has lead to progressively slows and prolongs the release of ciprofloxacin. Haemolysis percentage for the scaffolds was found to be 1.54, 1.52, 1.45, 1.82, and 1.75 for SC-0, SC- 1, SC-2, SC-3, and SC-4 respectively. The result showed higher degree of hemocompatibility of scaffolds and are suitable for the skin tissue engineering application. Fibroblast were isolated and cultured until the 90 % confluence was achieved. Scaffolds were

sterilized and cells were seeded 10^5 cellsper scaffold. Cell seeded scaffold were transferred inside CO_2 incubator under hypoxic condition (1% of oxygen) at 95% humidity and 37^0C for predetermined period of time. Fibroblasts attachment on the scaffolds was qualitatively evaluated by SEM and florescence microscopy. Effect of hypoxic condition on the growth of fibroblast over the period of time was studied using live& dead assay. SEM study shows that the uniform fibroblast attachment and growth over scaffolds, however cells differ in shape and number depending upon the nature of scaffold and availability of oxygen during cell culture. SEM image of SC-0 at 7th day clearly showed the attached fibroblast on surface with slightly altered morphology with lower number when compare to other scaffolds.SC-0 show slightly altered thread like projection because it does not provide oxygen for the fibroblast growth in the hypoxic environment during cell culture. This may be due to oxidative stress and free radical formation. Fibroblast growth, spreading, migration and proliferation were better from SC-1 to SC-4 which suggests increase oxygen releasing potential of scaffold promotes cell growth in hypoxic condition. Fluorescent microscopy using DAPI dye further strengthens the above made observation. SC-1 to SC-4 has continuous increasing cell number with SC-4 has highest number of cells due to their ability to generate oxygen during hypoxia that enables the scaffold to support the growth of cells inside scaffolds. Live & dead assay also showed that under hypoxic condition viability of cells was very low and viability of fibroblast increases as the oxygen generating potential of scaffold increases. MTT assay showed that the percentage viability of fibroblast value of SC-0 is significantly lower to other scaffolds due to low availability of oxygen during cell culture. Same interference is deduced from the DNA content estimation. On the bases of above mentioned morphological, physical and chemical and cell culture studies SC-4 was selected for further in vivo studies. Selected

scaffold was tested and found effective against *S. aureus* and *E. coli* showing antimicrobial nature of scaffold. In vivo study performed in rat model showed that oxygenating scaffold promotes the wound healing compare to the control and scaffold. Histological examination using Haematoxylin and Eosin staining of 15 days showed epidermis and dermis was found to be fully developed and ECM architecture with blood vessels and hair follicle was also observed compare to scaffold that has loosely organized ECM and control which still has less develop epidermal and dermal component along with ECM.

To conclude, quality of wound healing can be greatly improved by mediating the infection and hypoxia. Oxygen producing ciprofloxacin loaded collagen chitosan scaffold not only control the infection but it also provide the oxygen which is needed during the wound healing process.

Limitations in study-

2. For in vivo study higher numbers of rats were needed to perform at different environmental conditions.

3. During cell culture study stem cells can be used instead of primary cell culture.

4. Natural cross linker should be used instead of synthetic cross linker.

5. Modern technique such as 3 D printing can be used to achieve the near cell architecture instead of freeze-drying technology

Scope for further work:

With the designed and in vitro and in vivo evaluated of scaffold, following future studies can be carried out:

1. Scale-up of the culture studies involving 'cell seeded scaffold' in the bioreactor.
2. Reproducible *in vitro* studies with the human derived skin tissue cells.
3. Design and development of multilayered and multi cellular skin substitute.
4. Clinical trials need to be undertaken for oxygen ciprofloxacin loaded collagen-chitosan scaffold to decide the therapeutic potential on mass scale.
5. Addition of different growth factors inside PCL can be a suitable delivery approach for its slow and constant release form the scaffold.
6. Oxygenation scaffold with epidermal stem cells ESC), mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC) can be use to study the behavior under limited oxygen. In this way their capability to differentiate into outer cells can be tested and evaluated and can be used in future for deep skin tissue implantation.
7. Fibroblast and keratinocyte both can be used to prepare double layered skin tissue implant having different growth factors inside the PCL for slow, constant, and prolong release.