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Risk Assessment and Development of Collagen/PVA Nano-Fibrous E Spun Scaffolds for Improving Wound Healing Rate

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Keywords
Chronic wound, Nanofibrous system, Electrospinning, Risk Assessment, Wound Healing.

Abstract
Chronic wound is a major challenge worldwide. It imparts burden over Health-Related Quality of Life. Use of Natural Polymers has imparted to body's native Extracellular Matrix rejuvenating structure and elasto-mechanical characteristics in tissue regeneration and these novel nanofibric composites act far better compared to conventional formulation over loss of medicament (gel, ointment, cream) , adherence to the skin, ease of application and removal if any complications. Recent trends over the use of Electrospinning for the fabrication of fibrous composites ease the preparation with the economic benefits. Comparative market search states the leading gap over the Indian products for wound healing composites and need to be concerned to prioritize research in regenerative tissue scaffolds and its manufacturing techniques. Risk Assessment tool comprehends predictive variability and its impact towards the quality of product. Planning a control strategy for the process with the OFAT approach for the optimization of the process can give a higher quality product. The optimized batch was blended with the Poly (vinyl alcohol) and Collagen for the fabrication of nanofibers. Characterization states the high porous network was formed. This nano fibrous scaffolds were subjected to in-vivo animal model for wound healing. Based on results of requisite findings, it can be concluded that novel composites serves a promising approach over the compromised wound healing.

1. Introduction

Chronic wound does not progress through the healing process; chronic wound is a major challenge worldwide. [1,2] If the wound fails to the normal process of healing i.e. within 4 to 8 weeks, then are termed as chronic. Chronic Wounds during their lifetime and also leading to various complications like cellulitis, hemorrhage, gangrene, venous eczema and/or amputations.[3] Chronic wounds imparts significant humanistic as well as economic burdens, both at an individual's quality of life and healthcare costs. The humanistic burden represents the Health-Related Quality of Life (HRQoL) reflecting physical & psychological dimensions of health, assessed with economics to estimate the suffering from a condition or disease by the patients.[4–6] Categorization of wounds based on etiologies: Pressure Ulcers, Diabetic Ulcers, Artery and Venous Ulcers.[7] Survey in India, 62 million cases are reported of diabetes, Out of which approximately 25% of persons develop foot ulcer and 20% requires amputation. [8,9] The wound healing is a tedious process and involves four main stages.[10] The first stage is of Hemostasis; in first stage blood flow is reduced resulting in platelet aggregation and beginning of inflammation i.e. signs of rubor, calor, tumor and dolar. The secondary phase is the inflammatory phase, wherein, neutrophil starts

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to migrate and concentrate at wound site, the primary function of this stage is to remove bacterial contamination over the site. [11–13] The third phase is the proliferative phase, it starts once the wound is cleaned out.[14] The focus in this phase is to fill and cover the wound. The wound rebuilds with new tissue made up of collagen, ground tissue, glycosaminoglycans and the extra cellular matrix. The last phase is a remodeling phase, it starts when collagen is remodeled from form type III to form type I and the wound is closed completely.[15] The infectious wound presents complications like pus drainage, fever, foul odor, dull throbbing. A broad spectrum anti-infective drug can be used for the control of the wound infection.

Tissue engineering can act as a helpful tool in improving the healing rates. [16] Scaffolds are known protein based bioscience and they can act as a template for dermal regeneration and mimics extra cellular matrix components at the damaged site of skin.[17,18] Collagen is a natural biomatrix and is a protein available in the connective tissues such as cartilage, tendon, ligament, surrounding the cells, and forming the 3D cellular matrix of all tissue, giving characteristic texture, structure and shape to the skin.[19] Collagen being a constituent of connective tissue can play a major role in healing activity.[20] Scaffolds prepared with collagen is an important therapeutic option in chronic wound.[21,22] Different process are used to prepare scaffolds, Electrospinning is one of the method, which can be used to fabricate nanofibers.[23–26] Basically, the fibers are collected on a metal roller receptor and can be dispensed in the form of a porous polymeric composite or matrix or sheet. The electrospinning method is ease up scalability and is known for the continuous operation.[27] Comparatively, very less unit operations are associated using electrospinning with respect to other method like lyophilization. Moreover, Electrospinning technique for the preparation of fibers and polymeric matrix gives possibility of different dimensional fabrication of scaffolds. Electrospinning of a natural protein like collagen is a challenging task as the fiber strength and mechanical properties and use of organic solvents may not fall in the desired specification.[28,29] The use of a synthetic film former can solve this problem. Poly Vinyl Alcohol, Poly Urethane, Poly caprolactone are some of the examples of synthetic polymers which when used in combination with collagen can result into a polymeric matrix with robust mechanical properties.[30,31]

In the present study we have prepared nano fibers using combination of a natural polymer collagen and a synthetic polymer PVA. The Collagen /PVA spinning dispersion was prepared using aqueous vehicles and the use of different organic solvent was avoided keeping in mind the application of the prepared nano fibrous scaffolds in open chronic wounds.[32–34] The process was optimized and fabrication of the nano fibers was done with the fiber diameter in the range of 140 to 185 nm. Preclinical evaluation of the prepared nanofibers were done on wistar rats that shows significant healing property.

2. Materials and Methods

2.1 Materials

Collagen dough was gifted by MIL Laboratories Pvt. Ltd, Vadodara, Cold water soluble grade Poly Vinyl Alcohol was purchased from S D Fine Chem Ltd. Mumbai, The Electro Spinning unit; Super ES 0 was purchased from E- Spin Nanotech Pvt. Ltd. , IIT Kanpur. Distilled water (I.H.) was used in performing all the experiments.

2.2 Risk Assessment - Quality by Design Approach

The QbD approach is divided into the following steps, per the recommendations of the International Council of Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Setting forth the Quality Target Product Profile (QTPP), identifying the Critical Quality Attributes (CQAs), the Critical Material Attributes (CMAs), and Critical Process Parameters (CPPs), completing an initial risk assessment (RA), using a valid and reliable statistical Design of Experiment (DoE), creating the Design Space (DS), and defining the control strategy.To assess the hazards and determine the Risk Priority Number (RPN), an initial risk assessment (RA) was conducted by combining vulnerability and severity of the several parameters. Then, an assessment of the CQAs' and CMAs' and CPPs' interdependence was done. The relationships received one of three labels: low, medium, or high. In order to explore the cause-and-effect connections throughout knowledge space development, Ishikawa fish bone diagrams were made up for each stage of the experimental effort.

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2.3 Preparation of Nanofibers

Electro spinning unit Super ES 0 was used to prepare all the nanofibers considering one factor at a time approach (OFAT). 5 ml BD syringe was used with the needle having internal diameter of 23 gauge. A viscous 13 % W/V PVA solution was prepared in water by stirring and 8% w/v dispersion of Collagen in water was prepared. The dispersion was prepared using a high speed homogenizer. The prepared dispersion was mixed with PVA solution in equal ratio and used as an electrospinning polymeric feed. Other concentration of PVA were also prepared and tested but fiber formation with a good tensile property was not observed.

Different process parameters like flow rate, drum collector RPM, distance between needle to collector and voltage were optimized. The Super ES 0 can be operated maximum up to of 30 Kilovolt. The trials were taken by operating the machine at different voltages and keeping other parameters constant. No fibers were produced below 15 KV and at the voltage above 25 KV the fibers get widely distributed resulting in lower yield getting collected at the drum receptor. Thus, the optimized range for the voltage usage was found to be 15 to 25 KV.

The flow rate was optimized between 10 to 12 µL/min depending on the desired fiber diameter. Increased the flow rate resulted in droplet formation while the machine was operated in the optimized voltage range. The drum collector covered with aluminum foil was used as a receptor and the RPM of the spinning collector was operated between 250 to 400 RPM. Above 400 RPM fibers broke before it gets collected on the drum. The Drum collector to needle distance was kept constant at 15 cm for all the experiments.

2.4 Fourier Transform Infrared Spectrophotometry

Collagen and PVA pellets were prepared by the means of KBr press individually and the samples were analyzed using Fourier Transform Infrared Spectrophotometer (Burker, Germany) in the transmission mode with the region range of 4000-500 cm-1.

2.5 Thickness of the Scaffold

The thickness of the prepared scaffold was measured using a micrometer screw gauge. The thickness of the prepared scaffold was measured at total 9 points to know the uniformity of spraying pattern. As shown in the figure.

T1	T2	T3
T4	T5	T6
T7	T8	T9

Figure 1: Schematic Representation of Thickness evaluation points of nanofiber

2.6 Mechanical Strength

The mechanical strength of the sample was assessed using Texture Analyzer (TexturePro v2.1, Brookfield Engineering Laboratories, USA). 5cm * 5cm sheet was used as a sample. The sample was placed between the clamps and stretched at rate of 50mm/min. the study was performed at room temperature.

2.7 pH of the prepared scaffolds

1 cm2 film sample was cut and immersed in 10 ml deionized water. The assembly was incubated in orbital shaker for 36 hours and the pH was then measured by the means of pH meter (Elico India); immersing the electrode after calibration of system

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2.8 Folding Endurance

It gives the idea of firmness of any film. 25 cm2 of film sample is taken and folded until the film breaks and the counts are being noted as a part of evaluation.

2.9 Surface Morphology

The surface morphology study was performed using S-3400N (Hitachi) Scanning Electron Microscope at Sardar Vallabhbhai National Institute of Technology Surat. A small sheet of 5 mm * 5 mm size was used to perform the study. The images were taken with magnification of 10014, working distance of 6300 µm in greyscale color mode. The resulting image shows formation of nanofibers with diameters range 140 to 185 nm.

2.10 Percent Yield

The % Yield for the so formulated composite was noted by the mathematical formula given below:

$$\% \, Yield = Total\ weight\ of\ composite \times 100 / Total\ weight\ of\ solid\ contents$$

2.11 In-Vivo Analysis

Wound Excision model[35]

18 wistar rats were taken of either sex; and weighing up to 200-250 gms.

Three groups of Wistar rats were divided; 6 in each group.

Group 1: Control Group

2: Test Group Group

3: Standard Group

Each rats were pre-anesthetized by the means of diethyl ether and were shaved at the dorsal region. 10mm wound was created by the means of Biopsy punch. The rats are being treated based on the group and were caged individually. The wound contraction area was measured using transparency paper and marker at particular intervals.

2.12 Histological Analysis

Wound healing nature i.e. normal or granular healing was confirmed by the histological study. The tissue sample for group 1 and group 2 was taken in consideration for day 0 and day 15. Sample taken were stained using Haematoxylin-Eosin dye for visualization under optical microscope.

2.13 Stability Study

As per ICH Q1A (R2) guidelines, Stability study for the so formulated nanofibers was performed at room temperature (30°C ± 2°C/75%RH ± 5%RH) and accelerated condition (40°C ± 2°C/75%RH ± 5%RH) for 6 months. Tensile Strength were noted as a part of stability aspects.

3. Result and Discussion

Electrospinning being a comparatively new process, the factors affecting the quality related to material and process were studied. The study's main objective was to prepare nano fibers that can improve wound healing rate. A risk assessment study was performed to show the impact of selected CMA and CPP on each selected CQAs. The RA matrix is color coded as shown in table 1, Ishikawa diagram was also prepared as shown in figure 2.

Table 1: Risk Assessment Matrix

CQA	CMAs			CPPs				
	Natural Polymer	Synthetic Polymer	Ratio of Natural to Synthetic Polymer	Flowrate	Applied Voltage	RPM of Collector	Tip to collector distance	Type of Collector
Healing activity	HIGH	LOW	HIGH	LOW	LOW	LOW	LOW	LOW

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Mechanical strength	HIGH	HIGH	HIGH	HIGH	HIGH	HIGH	HIGH	HIGH
Diameter of nanofibers	LOW	LOW	LOW	HIGH	HIGH	HIGH	HIGH	HIGH
Homogenous thickness	MEDIUM	MEDIUM	MEDIUM	HIGH	HIGH	HIGH	MEDIUM	HIGH

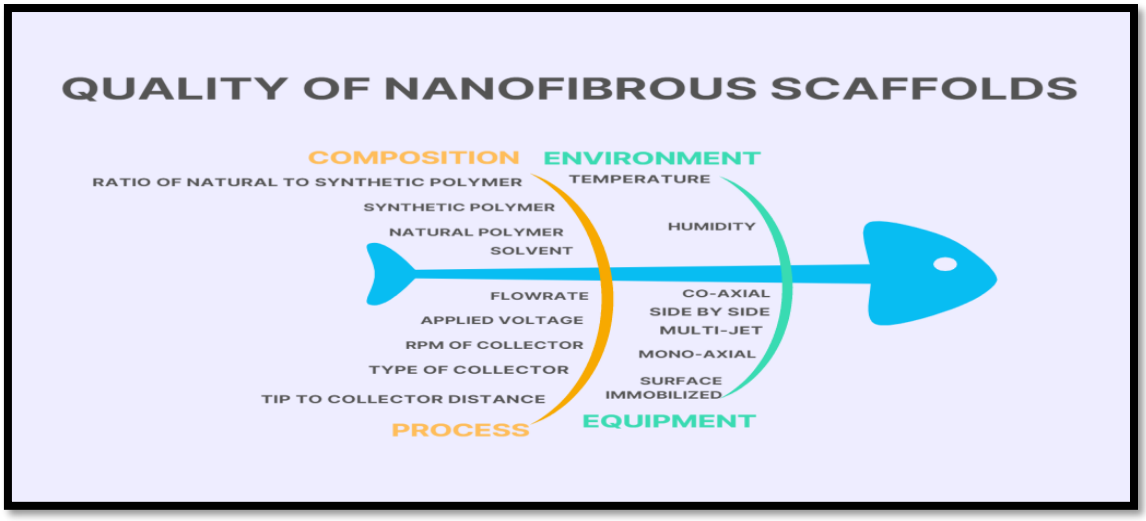

Figure 2: Ishikawa diagram

3.1 Fourier Transform Infrared Spectrophotometry

The overlay plot from FTIR determines the presence of functional group at specific wavelength (cm-1). The plot for prepared nanofibrous gives up no interaction with respect to the polymers used. It was found to be identical with respect to individual plots of Poly vinyl alcohol and Collagen representing the presence of functional groups.

3.2 Thickness of scaffolds

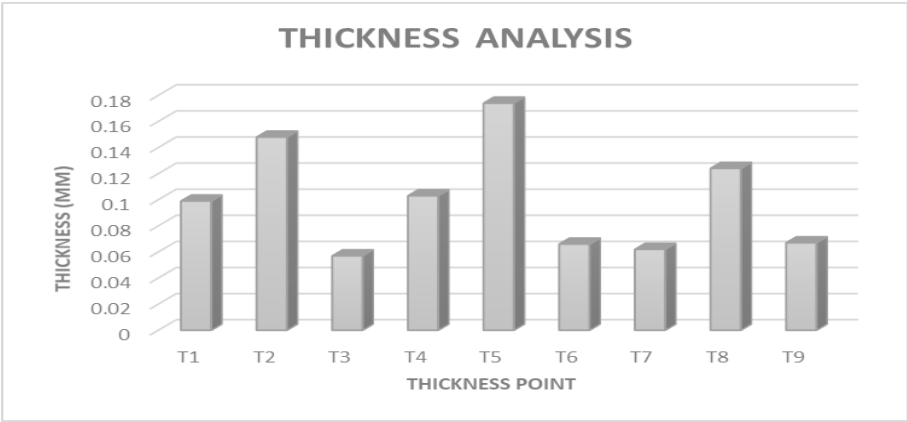

Figure 3: Evaluation data for thickness of nanofiber

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The thickness at different point shows the distribution patter or the spray pattern of electrospinning. The higher thickness was found at the center points determining the straight spray pattern at the 15 to 25 KV current.

3.3 Mechanical Strength

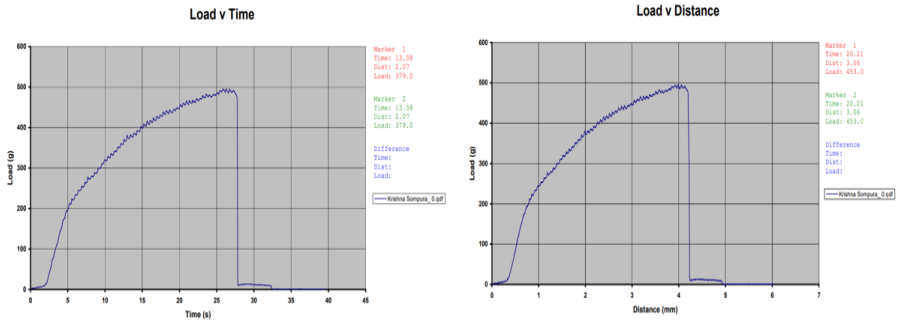

Figure 4: Tensile report of the nanofibers

The flexibility of the nanofibers can be measured by the means of tensile analysis which out performed by Texture Analyzer at Nirma Institute of Pharmacy. The prepared nanofibrous was found to bare up to more than 400 gm of load (force) with the displacement of 4 mm under constant temperature and pressure.

3.4 pH of Nanofibers

pH claims an important note once used for open wound or the topical cases. The ideal pH range of wounds was found to be 7.15 to 8.9.[36] The reading was performed in triplicates (n=3). The result found to be near with respect to the range 6.9 ± 0.08.

3.5 Folding Endurance

The firmness was found to be 30 – 32 numbers for the 25 cm2 dimension based on the conventional analysis of folding endurance at room temperature.

3.6 Surface Morphology

The surface morphology of the nanofibers is shown in Fig. The morphology possessed a well interconnected porous fibrous network structure with relatively porosity. The fiber diameter was found to be 140 – 183 nm under 5 µm scaling magnification.

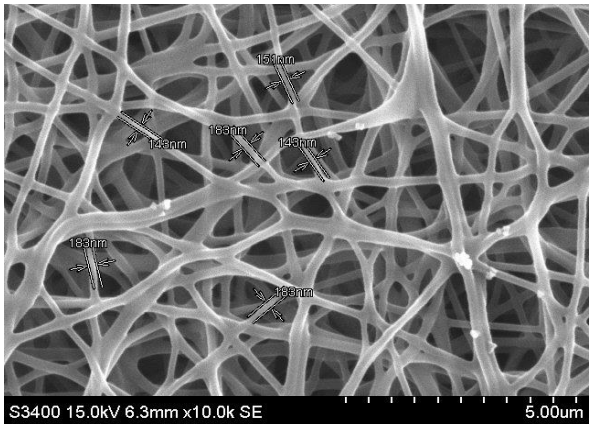

Figure 5: SEM image of the nanofiber determining the diameter of nanofibers

3.7 Percent Yield:

Based on the mathematical formula from 2.9, triplicate reading was to be taken claiming an observation of 71.35 % ± 1.65**.** Furthermore, the loss was predictable of 30% due to change on spray pattern because of voltage difference.

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3.8 In-vivo analysis

The Healing pattern is found to be identical towards test formulation. The time span was observed for 15 days. The area of contraction was measured manually by the means of transparent paper and marker placing on the wound area. The data was statistically compared.

Table 2: Wound Contraction Area (mm^2)

WOUND CONTRACTION AREA (mm^2)			
DAY	NORMAL GROUP	TEST GROUP	STANDARD GROUP
0	78.5 ±0.00	78.5 ±0.00^{ns}	78.5± 0.00^{ns}
5	57.2 ±0.71	59.8 ±1.41 ^{ns}	63.2± 0.21*
10	13.6 ±1.41	38.0 ±0.71***	41.7± 0.71***
15	0.2 ±0.22	0.1 ±0.00 ^{ns}	7.5± 0.05**

[n=6, Mean ±SDM; p > 0.05 ns, p < 0.05 *, p < 0.001 ***]

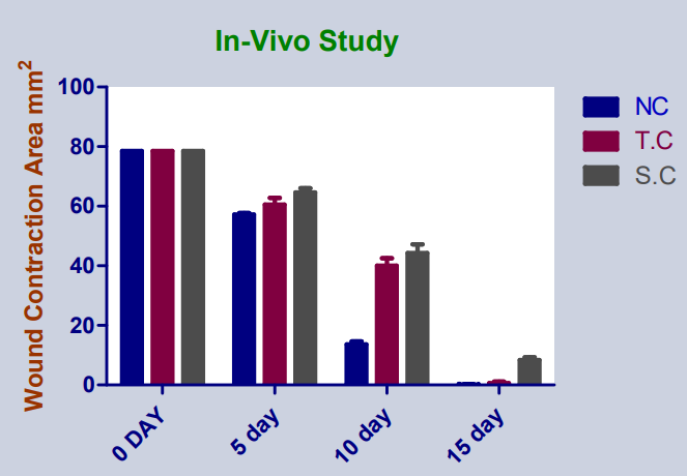

Figure 6: Graphical Representation of In-Vivo Study on Wistar rats

	DAY 0	DAY 5	DAY 10
TEST GROUP			

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STANDARD GROUP			
CONTROL GROUP			

Figure 7: Wound Contraction Images for the nanofibers by preclinical study

3.9 Histology Analysis

Histology was performed for the Normal Skin and Normal Wound after 15 days. The Images shows the presence of the different skin layer claiming the healing to be normal. Presence of well differentiated different layers of skin was observed. The profound epidermis, hypodermis, hair follicles, dermis and sebaceous gland was observed.

DAY 0,	DAY 15
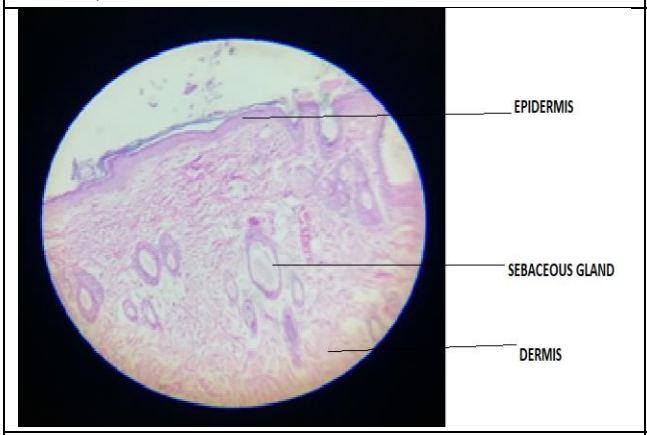	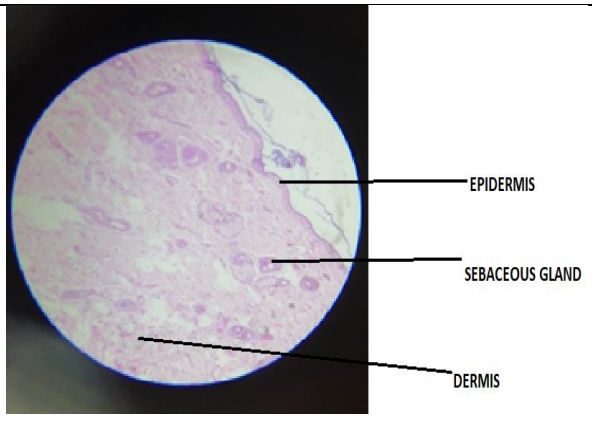
Figure 8: Histological image of animal skin at day 0, magnification 10X	**Figure 9:** Histological image of animal skin at day 15, magnification 10X

3.10 Stability Analysis

Stability analysis was carried out for nanofibres formulation according to International Conference on Harmonization (ICH) guidelines. A sufficient dimension of nanofibrous sheet was kept at room temperature i.e. at 25° C ± 2° C/60% ± 5% RH, refrigerator i.e. at 5° C± 3° C and accelerated conditions (40° C±5° C/75±5%RH) for 1,3,6 month. After 1, 3, 6 month samples were evaluated for folding endurance, pH, and tensile analysis. The pH was found to be 6.9 ± 0.07 with the robust

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mechanistic properties claimed by folding endurance of more than 30 folds and tensile analysis baring load (force) of 350 gm with the displacement of 4 mm. the so formulated nanofibers was found to be flexible enough.

4. Conclusion

The nano fibers were fabricated using e spin technology. The PVA/Collagen ratio was screened in terms of mechanical strength and by ease of detachment from the foil. The e spun nanofibers were composed of PVA (13 % w/w) and collagen (8 % w/w) in 1:1 ratio. Risk Assessment was carried out and showcased in Ishikawa diagram for the vulnerability of the Product. The process parameters of e spinning were optimized by OFAT (One Factor at Time) Approach. The prepared e spun scaffolds were characterized and evaluated for different parameters like, compatibility by FTIR that found satisfactory. The thickness of the scaffold was measured using a micrometer screw gauge at 9 different points that suggests more uniform spraying pattern at the center. The pH of scaffolds were measured after dissolving it in de ionized water and was reported between 6.5 to 7.5. The flexibility of the prepared scaffolds were measured by texture analyzer, suggesting breaking at 400 gm load with the displacement of 4 mm. Folding endurance was around 30-32 folds. The surface morphology was studied using scanning electron microscopy S-3400 N (Hitachi) and confirms loading of multiparticulates and the diameter of the nano fibers was between 140 to 185 nm at 5 µm magnification scale. In vivo wound healing study was performed on wistar rats divided into 3 different groups. The comparative results shows significant healing compared to normal control group. Histological study was also performed that shows normal wound healing and skin re epithelialization. The accelerated and real time stability studies were performed at 40°C ± 2°C temperature and 75% R.H.± 5% shows robust and stable system in terms of pH, folding endurance and Tensile Strength. As a consequence, activity can be achieved over impaired wound healing as desired in chronic wounds.

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