ORIGINAL ARTICLE

Ciprofloxacin-loaded Gelatin Microspheres Impregnated Collagen Scaffold for Augmentation of Infected Soft Tissue

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Abstract

Introduction: The development of bioactive scaffold to regenerate/augment the connective tissue in infected dermal wounds is still challenging in the field of regenerative medicine and tissue engineering. This is why protein based biomaterial scaffolds that mimic extracellular matrix and acts as the template for the regeneration of dermis and epidermis at the wound site are widely adopted for wound regeneration and soft tissue augmentation. Collagen, the most prominent biomaterials mostly enhance wound healing and maturation of collagen fibers by providing a scaffold for the better rapid transition to tissue with mature, aligned collagen fibers at the wound site. However, in the case of infected wound, wound pathogens secrete enzymes such as bacterial gelatinase and collagenase degrade collagen scaffold as collagen itself is protein. So that, it is necessary to incorporate the antimicrobial agents into the collagen scaffold which can deliver the drugs in a controlled manner by encapsulation of drugs into the vehicles and could be developed to control and eradicate the wound pathogens and supports regeneration faster. **Methods:** The wound care system which contains a porous collagen scaffold impregnated with ciprofloxacin-loaded gelatin microspheres and has a capable of delivering the ciprofloxacin in a controlled manner at the wound site is developed. The degradation of gelatin microspheres by enzymes secreted by wound pathogens in the wound site ensures drug release at the wound site, and collagen scaffold also supports skin regeneration. The morphological studies of microspheres and microsphere-impregnated collagen are evaluated with scanning electron microscopy. **Results and Discussion:** The encapsulation efficiency of the drug in gelatin microspheres is ~78.6%. *In vitro* drug release profile confirms that ~27% of drug burst released within 5 h followed by controlled release up to 2 days. *In vitro* antimicrobial evaluation of the scaffold showed the significant zone of inhibition against pseudomonas pathogens. *In vivo* study of the wound care system showed that gelatin microspheres impregnated in collagen scaffold heal full thickness wound in 12 days, whereas antibiotic incorporated collagen sponge and plain collagen sponge heals in 16 and 20 days, respectively. The wound closure in the animals and histological analysis of granulation tissue confirmed that epidermis and dermis regenerated at the wound site. **Conclusion:** This investigation suggests that the incorporation of ciprofloxacin-loaded gelatin microspheres impregnated porous collagen scaffold bestows sustained release of ciprofloxacin when gelatin degrades in the infected wound environment, thereby formulating an effective wound care system.

Key words: Controlled release, drug encapsulation, gelatin microspheres, *in-vivo* studies, wound infections and ciprofloxacin

INTRODUCTION

The microencapsulation of either antimicrobial agents or biotherapeutic molecules such as growth factors provides to overcome various limitations in the controlled release formulation of wound care system such as low solubility, high potency, and/or poor stability of many of these drugs. The

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microencapsulated-based drug delivery can impact efficacy and potential for commercialization as much as the nature of the drug itself. Moreover, the microencapsulation facilitates biocompatible could provide high bioavailability at the site of injury and are capable of sustained release for long periods into the wound environment.[1,2] There are certain limitations available in the existing wound care system such as lack of controlled delivery, microbial degradation of collagen-based wound dressings by wound pathogens at the wound site.[3] To overcome these limitations, an effective wound care system is developed based on microsphere-based controlled delivery of a potent and broad spectrum antimicrobial agent into an infected wound environment.[3] In this novel wound care system, the microsphere-based collagen scaffold provides to control effectively the infection at the wound site and fast the wound regeneration including dermis and epidermis. The regeneration of connective tissue at the injured soft tissue is still exigent. The presence of bacterial pathogens at the wound site delays wound closure and regeneration of connective tissue as well as degrades various extracellular matrix like collagen and elastin and also produce high inflammation matrix at the wound site by its native enzymes such as microbial collagenase and elastase.[4-6]

The scaffolds made from protein-based biomaterials acts as a template for dermal regeneration and mimics extracellular matrix at the damaged site.[7] Among all the protein-based biomaterials, collagen is the predominant natural polymer and most abundant protein in the connective tissue such as cartilage, tendon, and ligament, surrounds the cells, and forms the 3D cellular matrix of all tissue, giving each its characteristic structure, texture, and shape.[8] Collagen is of particular interest as a natural polymer for drug delivery since it is a major natural constituent of connective tissue and a major structural protein of any organ. Having advantages such as biocompatible and non-toxic and well-documented structural, physical, chemical, biological and immunological properties, additionally, drug release kinetics can be influenced by modification of the matrix characteristics (porosity and density) or by different chemical treatment regimens affecting its degradation rate.[9]

Past decade, collagen-based biomaterials are used as wound dressings for dermal regeneration for wound management. In the case of infected dermal wound healing, the collagen scaffold is not able to control the infection and to eradicate the wound pathogens at the wound environment. Because, collagen itself is a protein in nature and not used in highly infected wounds, wound pathogens degrade collagens effectively and use this as substrates for their growth. The antimicrobial agents incorporated in collagen biomaterials needs not only to stabilize the collagen biomaterial and but also to eradicate wound pathogens at the infected wound site. The sustained or controlled release of antimicrobial agents into the wound environment provides a prolonged antimicrobial activity to eradicate pathogens rapidly and fasts the wound healing. As a result, the dermis and epidermis in the wound environment could be regenerated fast and effectively.[10,11] In our fabrication of scaffold for wound repair, the combination of two different protein materials used for constructing the scaffold could accelerate the formation and regeneration of connective tissue in the infected wound. Moreover, one of the protein materials (gelatin) can act as drug carrier and another scaffold (collagen) as a template is for regeneration of dermis and epidermis in the wound environment.

A possible method to deliver the drug in a controlled manner is by entrapping antimicrobial agents loaded protein-based microspheres incorporated in the collagen matrix.[12] Among the protein-based polymers, gelatin is a denatured collagen which also supports cell fate process and effectively encapsulates drug for fabrication of microspheres. Gelatin is a chosen biomaterial for fabrication of microspheres. Because it is a denatured collagen in nature and susceptible to degradation by moist environment and microbial enzymatic degradation at the wound site.[13] The release of a potent antimicrobial agent from the microsphere is done by degradation and level of infection at the wound site. Therefore, the more infected wound contains more secreted microbial enzymes available at the wound site and the wound environment is moister. As a consequence, the amount of drug released from the microspheres depends on the wound environment and level of infection at the wound site. The chosen drug is ciprofloxacin for drug delivery to wound surface. Ciprofloxacin (1-cyclopropyl-6-fluro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) is chosen as a model antimicrobial agent due to its broad spectrum activity and also the presence of reactive amine and carboxylic acid as functional groups. It has good penetration in tissues and treatment of infections in bones and soft tissues and also has the advantages of antimicrobial activity against beta-lactam-resistant organisms. This antibiotic is the leader among third-generation fluoroquinolones with a broad spectrum of antibacterial activity covering both Gram-positive and Gram-negative bacteria and has a good penetration in most. The bactericidal activity generated by fluoroquinolones is caused by their inhibition of bacterial DNA gyrase and topoisomerase IV enzymes. DNA gyrase is essential for the replication, transcription, and repair of bacterial DNA and topoisomerase IV is involved in the partitioning of chromosomal DNA during cell wall division. By inhibiting those enzymes, fluoroquinolones keep cellular bacterial DNA in a supercoiled state, thereby preventing bacterial replication [Figure 1].[14-16]

Although collagen is a protein and capable to regenerate the tissue at the wound site, it is not suitable for treating the infected dermal wound, because wound pathogens could degrade the collagen and the degraded hydrolysates can act as a nutrient for wound pathogens. As a consequence, the wound closure delayed and the biosynthesis of extracellular matrix is not progressed at the wound site.[17] To overcome this problem, the collagen scaffold is engineered by impregnating antimicrobial agents into the scaffold or incorporation of

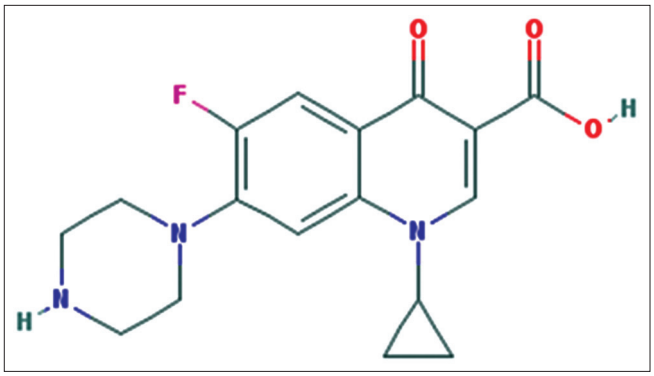

Figure 1: Structure of ciprofloxacin

bioactive microspheres into the scaffold. The engineered collagen scaffold not only provide the faster regeneration of the wound but also effectively eradicates the wound pathogens at the wound site by sustained release of antimicrobial agents into the wound environment. This work describes the development and characterization of a reconstituted collagen scaffold impregnated with ciprofloxacin-loaded gelatin microspheres, a capable of delivering ciprofloxacin in a sustained release manner to combat the wound pathogens at the wound environment and fast the wound closure. This would be a probable solution to effectively manage wound infection, with the active participation of wound healing drug delivery scaffold in the healing process, and lessen the frequency of routine dressing change.

MATERIALS AND METHODS

The biomaterials for fabrication of scaffold

The pure Type 1 Gelatin (Sigma Chemicals) is used for fabrication of antimicrobial agents incorporated microspheres for controlled release. The Type 1 collagen extracted from bovine tendons is used for fabrication of macroporous scaffold as a template for soft tissue repair.

Extraction of Type 1 collagen scaffold

The preparation of collagen from bovine tendon was done according to the method developed by, Bio Products Lab, Central Leather Research Institute, Chennai, India. The bovine tendons were collected from the local slaughter house, Chennai and washed thoroughly with distilled water to remove residual blood. The minced tissues were washed using a nonionic surfactant. The washed tissues were suspended in 2% sodium peroxide solution for swelling and then washed with distilled water. The coagulated collagen was then suspended in phosphate buffer solution of pH 8.5 and treated overnight with trypsin (0.5% w/w). The tissue was again washed in distilled water to remove the enzyme and dissolved salts. The coagulated tissue was swollen again in distilled water after adjusting the pH of water to 2.5 with hydrochloric acid and treated with pepsin (0.3% w/w), overnight. After the pepsin treatment, tissues were washed repeatedly in water to remove the enzyme. The coagulated collagen was dissolved in millipore water acidified to pH 3.5 using HCl to get pure collagen solution. The undissolved proteins were removed by centrifugation at 10,000 rpm for 30 min. All the above operations were performed at a temperature of 15°C ± 2°C. Purity of collagen was checked by various physicochemical characterization and biochemical studies.[18]

Preparation of gelatin microspheres

Gelatin microspheres were prepared by the microencapsulation method. 10 ml of an aqueous solution of ciprofloxacin (7.5 wt %) and acidic gelatin (10 at % preheated to 40°C) was added dropwise into 375 ml of olive oil while stirring at 420 rpm at 40°C for 10 min to yield a water-in-oil emulsion. The emulsion temperature was decreased to 15°C, followed by further stirring for 30 min to allow for natural gelation of the gelatin aqueous solution. Acetone (100 ml) was added to the emulsion and stirring was continued for 1 h. Then, 30 ml of glutaraldehyde (GA) added and saturated. Toluene was added to the suspension/emulsion system. Stirring was continued for 4 h to allow the cross-linking of gelatin microspheres to be completed. The resulting microspheres were washed three times with acetone and recovered by centrifugation (5000 rpm, 4°C, and 5 min). The washed microspheres were placed in 100 ml of 100 mm glycine aqueous solution containing tween 80 (0.1 at %), followed by agitation at 37°C for 1 h to block the residual aldehyde groups of unreacted GA. Microspheres were then washed with 30 ml of cold isopropyl alcohol at 5°C overnight. Microspheres were then freeze dried at 37°C and stored in colored glass containers.[19]

Characterization of gelatin microspheres

Determination of drug loading and encapsulation efficiency

Drug-loaded microspheres (100 mg) were digested with 10 ml of 1 N sodium hydroxide at room temperature for 12 h. The solution was filtered and analyzed at 278 nm using high-performance liquid chromatography (HPLC), to determine the amount of ciprofloxacin present in the microspheres. The drug loading in microspheres was estimated using the formula:

$$L = \frac{Q_m}{W_m} * 100$$

Where L is the percentage loading of microspheres,

Q_m is the quantity of ciprofloxacin present in W_m g of microspheres.

The amount of ciprofloxacin encapsulated in the microspheres was determined using the formula:

$$E = \frac{Q_p}{Q_t} * 100$$

Where E is the percentage encapsulation of microspheres, Q_p the quantity of drug encapsulated in microspheres (g) and Q_t is the quantity of ciprofloxacin added for encapsulation (g).

Particle size analysis

The size analysis of microspheres was performed by laser diffraction using a Malvern Hydro 2000 SM particle size analyzer (Malvern Instruments, Worcestershire, UK). The dried powder was dispersed in isopropyl alcohol and vortexed for 10 s. The particulate dispersion obtained was added to the sample dispersion unit containing stirrer and stirred to minimize the interparticle interactions; the laser obscuration range was maintained between 10% and 20%. The instrument was set to measure the sample 3 times at a rate of 90 snaps (or counts) per second. The sample was counted three times and average volume mean diameter was obtained.

Scanning electron microscopy (SEM)

The sample for the SEM analysis was prepared by sprinkling the microspheres one side of the double adhesive stub. The stub was then coated with gold using Joel JFC 1100 sputter coater. The SEM analysis of the microspheres was carried out by using Jeol JSM 5300, Japan. The microspheres were viewed at an accelerating voltage of 15-20 kV.

Differential scanning calorimetry (DSC)

DSC of ciprofloxacin and microspheres were performed using NETZSCH DSC 204. The instrument was calibrated with indium. All the samples (≈5 mg) were heated in aluminum pans using dry nitrogen as the effluent gas. The analysis was performed with a heating range of 50-200°C and at a rate of 10°C/min.

Preparation of collagen scaffolds from Type 1 collagen

About 1% collagen solution was prepared in acidified water using acetic acid. To this 400 µl of Triton X-100, non-ionic surfactant was added and agitated for few minutes to attain homogeneity. It was poured into the trough and allowed to dry in the air in a dust free chamber or freeze dry the collagen suspension. The plain collagen scaffolds used as one of the controls in the experiment.[20]

Incorporation of ciprofloxacin into the collagen scaffold

After getting homogenized collagen preparation, a known amount of ciprofloxacin (0.5 mg) was added and stirred well and poured in the trough. Since the minimum inhibitory concentration of ciprofloxacin against *Staphylococcus aureus* ATCC 29213 is 0.12-0.5 (µg/mL) and *Pseudomonas aeruginosa* ATCC 27853 is 0.25-1.0 (µg/mL), the amount of drug to be added in the collagen scaffold is 10 times of minimum inhibitory concentration value of drug per cm^2 of the scaffold. The thickness of prepared collagen scaffold is 2 ± 0.5 mm.[21-23]

Fabrication of gelatin microspheres impregnated collagen (GMC) scaffold

Gelatin microspheres (5 mg) impregnated with ciprofloxacin was suspended in 0.4 ml saline. The gelatin microsphere suspension was uniformly injected into several portions of the inner collagen sponge. The ciprofloxacin doses were 5 mg/scaffold. The doses used in this study were based on our own preliminary dose-response studies.[12,24,25]

In vitro release of ciprofloxacin from gelatin microspheres incorporated collagen Scaffold

In vitro release of ciprofloxacin incorporated gelatin microspheres (5 mg) in collagen sponge was carried out at 37.1°C in phosphate buffered saline (PBS) (50 ml) pH 7.4. The release medium was collected at predetermined time intervals, and replaced with a fresh buffer of PBS (1 ml) each time. The collected samples were filtered through a 0.45 mm Millipore filter. The amount of ciprofloxacin released was then measured at 278 nm using a Shimadzu ultraviolet (UV)-2100S spectrophotometer and HPLC with an UV detector. The HPLC equipment comprised a solvent delivery system (model PU-980, Jasco), Rheodyne injector (model 7125, Cotati). The analytic column was a Novapak C18 cartridge with a 4 µ particle size at 100 mm × 8 mm internal diameter (Waters Chromatography and Division) protected with a precolumn containing a Novapak C18 insert. The mobile phase consisted of methanol: acetonitrile:0.4 M citric acid (3:1:10, v/v/v), and the flow rate was 1 ml/min at ambient temperature.

Sterilization of drug incorporated collagen scaffold

Ciprofloxacin incorporated collagen scaffold and plain collagen scaffold are sterilized by ethylene oxide sterilization.

Agar diffusion test for *in vitro* antimicrobial activity

Microorganisms tested

The bacterial strain *P. aeruginosa* ATCC 27853 collected from the King Institute, Chennai, India. Soya Bean Casein Digest Broth (Hi-Media Pvt. Ltd., Bombay, India) was used for the test bacterial strains. Bacterial cultures, freshly grown at 37°C were appropriately diluted in sterile normal saline solution to obtain the cell suspension at 10^5 CFU/ml.

Antimicrobial properties of gelatin microsphere incorporated collagen scaffold (10 mm diameter) were tested on agar plates inoculated with a mixed culture of *S. aureus* and *P. aeruginosa* in accordance with the Kirby-Bauer disk diffusion test.

In vivo evaluation of the bioactive scaffolds

Male Wistar albino rats weighing 150-200 g were used for this study. The animals were fed with commercial pellet diet (Hindustan Lever, Bangalore, India) and had free access to water. The animal experiment was performed according to the Institute's ethical committee approval and guidelines (466/01/a/CPCSEA). The wound creation was done,[7] and the experimental rats were dressed with formulated dressing, plain collagen scaffold, and collagen scaffold with antibiotic while the control group was dressed only with gauss dressing. All rats were given regular changes on every day while the formulated dressing and the antibiotic dressing were changed once in 2 days [Table 1].

Wound contraction

The reduction in the size of the wound was measured at every 4 days intervals and given as a percentage of wound contraction. The following formula was used to calculate the percentage of wound reduction: The percentage of wound reduction/wound closure is given by:

$$\frac{\text{Wound area day 0 - wound area day } (n)}{\text{Wound area day 0}} \times 100$$

$n = 4^{th}$, 8^{th}, 12^{th} and 16^{th} day

Histological analysis

Tissues collected at different intervals were transferred to 10% neutral buffered formalin for 24 h at 4°C. The formalin fixed tissues were dehydrated through grades of alcohol and cleared in xylene and then embedded in paraffin wax (58-60°C melting point). The molds were labeled and stored until use. The deparaffinized sections were stained with hematoxylin following counterstained with eosin. Masson's trichrome staining was done for all the samples of all the time points to observe collagen deposit in the granulated tissue.[26]

Table 1: Groups for *in vivo* studies

Names of the group	Number of male albino Wister rats involved (*n*=6)
Open wound groups	24
Ciprofloxacin-loaded GMC scaffold	24
Ciprofloxacin-loaded collagen scaffold	24
Plain collagen scaffold	24
Total animals	112

GMC: Gelatin microspheres impregnated collagen

Bacteriological examination of granulated tissue

Superficial muscles/granulated tissues were excised on the days 4, 8, 12, and 16. 1 mg of excised tissue was placed in 10 ml of sterile saline, vortex for few minutes and the total bacterial count was analyzed by serial dilution method.[22]

Statistical analysis

All results were expressed as mean ± standard deviations, and the results were compared statistically by Student's independent *t*-test using SPPS software (student version 7.01). The $P < 0.05$ was considered statistically significant [Figure 2].

RESULT AND DISCUSSION

The skin is the largest organ system of the body; an organ of protection plays a role in regulation and sensory reception and is involved in immunological surveillance. The primary function of the skin is to serve as a protective barrier against the environment. The physical/chemical/biological injury of skin leads to the progressive destruction of soft tissue and formation of the wound with infection.[27] The infected wound is a synthetic environment in which a number of cellular processes are interlinked and leading to the regeneration of dermis and epidermis. Tissue injury involving cell death, destruction of extracellular connective tissue components and loss of blood vessel integrity automatically triggers the wound healing process.[28]

In general, infections at the wound site delay healing process and wound pathogens inhibit the collagen synthesis at the wound site. Wound pathogens are capable of secreting the enzymes which degrade extracellular matrix at the wound site. The development of a wound infection is dependent on the pathogenicity and virulence of the microorganism and immunocompetence of the host. In wounds, extracellular infection is more common than intracellular infection and many pathogens rely on the production of extracellular enzymes to invade deep into the soft tissue. The progressive infection at the wound site leads to the formation of biofilm. Biofilms are communities of microbial cells, attaches to surfaces and encased in slime. This offers protection against phagocytosis, antibiotics and antimicrobial agents. Biofilms in chronic wounds may be linked to failure to heal and regeneration of dermis and epidermis.[4,5,16]

The principal pathogens associated with wound infections are *S. aureus*, *Streptococcus* species anaerobes, and *P. aeruginosa*.

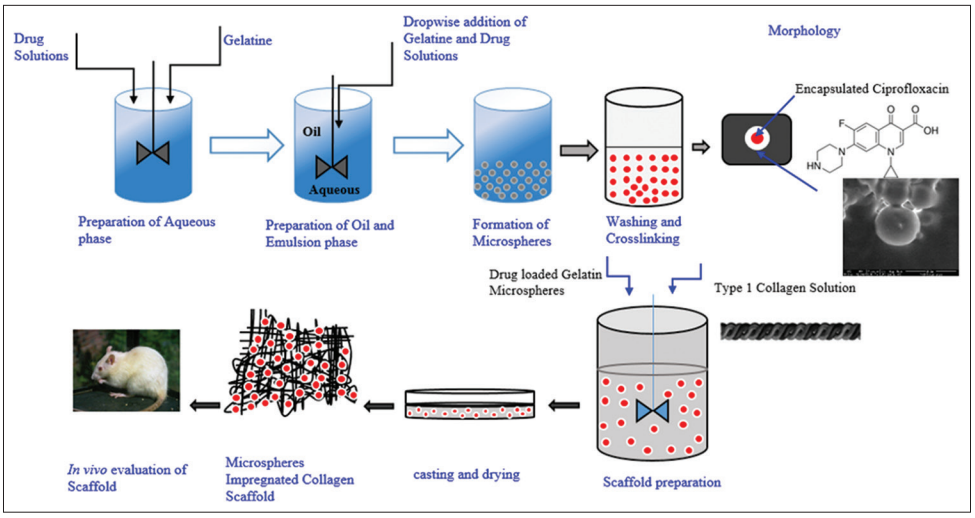

Figure 2: Preparation of gelatin microspheres incorporated collagen scaffold

S. aureus and *P. aeruginosa* secrete extracellular enzymes of collagenase and elastase. Collagenase hydrolyzes collagen in necrotic tissue but does not attack collagen in healthy tissue. Elastase could degrade elastin in the extracellular matrix of the soft tissue. In addition, *P. aeruginosa* secretes numerous protease enzymes which degrade many proteins in the wound site. As a consequence, wound closure is delayed.[4,5]

The topical administration of antimicrobial or wound healing agents in the form of solution or gel directly to the wound site will diffuse rapidly and gets eliminated from the site, thus local effective concentration cannot be maintained and results delayed wound healing. The controlled/sustained release formulations can be used to reduce the amount of drug necessary to cause the same therapeutic effect in patients and also to eradicate microbial infections.[10]

Natural polymers are being used in the field of regenerative medicine for the treatment of various tissue repair and the best alternative for synthetic polymers. Mostly, natural polymers could mimic the extracellular matrix and provide a microenvironment of cell fate process which is essential for tissue regeneration. In the present investigation, gelatin is used as a base biomaterial for encapsulation of antimicrobial agents. The drug loaded gelatin microspheres are prepared by microencapsulation method and then impregnated with collagen scaffold to make an effective drug delivery system for infected wound healing.

Collagen is the most prominent biomaterial among natural polymers and is biodegradable, biocompatible, non-immunogenic, and suitable for easy modification of the side chain of an amino acid of the collagen for various functionality. Moreover, it plays an important role in maintaining the biological and structural integrity of the extracellular matrix in soft and hard tissue. Moreover, the bovine Type 1 collagen has low antigenicity and outstanding biocompatibility with most endogenous tissue and used for various surgical repair. Therefore, Collagen-based wound scaffold used to treat burns, wounds, and ulcers. The extracted Type 1 collagen from bovine tendons was proved mild antigenic, tissue biocompatibility, and suitable for developing scaffolds for tissue engineering application. The prepared porous collagen scaffold support cell fate process and increase the surface area of biomaterial which helps to increase the cell and matrix interaction.

The collagen-based drug delivery system with effectively realizing ciprofloxacin into the wound site is developed for effective eradication of wound pathogens and impeccable regeneration potential of injured tissue. In this drug delivery system, the gelatin microspheres act as drug carrier and collagen scaffold supports for skin regeneration and act as a reservoir for ciprofloxacin-loaded gelatin microspheres. Collagen has been proved as a very attractive material for wound regeneration. Collagen scaffold has the ability to absorb large quantities of wound fluid. The porous nature of collagen scaffold is sufficiently large to absorb and deliver drug sustained manner. An ideal drug delivery system is one that delivers a sufficient quantity of drug at the site of an infected area in the wound environment and prevents further bacterial proliferation at the wound site. In controlled release formulations, collagen scaffold immediately placed on on the release medium, an initial large bolus of drug is released before the release rate reaches a stable profile. This phenomenon is typically referred to as "burst release." When it comes with *in vivo* studies, Burst release could help to arrest wound pathogen growth and followed by the controlled release of antimicrobial agents to the wound site eradicate wound pathogens and enhanced the regeneration of soft tissue and leads to better wound closure.

Collagen itself is protein in nature. The plain collagen scaffold is highly susceptible to microbial degradation by wound pathogens in the wound site. Therefore, it is essential to incorporate anti-microbial agents into the collagen scaffold and it provides a guard from microbial degradation and offers a sustained release to the wound environment. Past decade, collagen based biomaterials used for soft tissue regeneration. Antimicrobial agents must incorporate with

collagen biomaterials for infected dermal wounds. Because of collagen itself protein, wound pathogens utilize collagen as a substrate for their growth and may cause biofilm formation at the wound site. Gelatin microspheres are used as a drug carrier. Gelatin also has the support for tissue regeneration and has a capacity of drug entrapment. The combination of collagen and gelatin scaffold may influence better dermal regeneration at the injury site.

Characterization of gelatin microspheres

The yield of microspheres based on this protocol is 88.2%. As shown in Table 2, the percentage of yield was 88.2%, which indicated a low loss of microspheres during preparation and recovery. The estimation of drug content and release profile is done by UV-visible spectrophotometer and HPLC partially as given in supplementary information. The formulated microspheres were with free flowing yellow colored powder in nature. The olive oil was found to produce spherical microsphere without aggregation. The GA-saturated toluene solution was used to cross-link and stabilize the gelatin microspheres. After stabilization, the microspheres were agitated in 5 ml of 10 mM aqueous glycine solution at 37°C for 1 h to block the residual aldehyde groups on unreacted GA. The stirring speed and gelatin/drug ratio were optimized by observing the particle size under a microscope.

Table 2 confirms the performance of the microencapsulation process for the preparation of ciprofloxacin-loaded gelatin microspheres and this process achieved ~78.6% encapsulation of the antimicrobial agent in the microspheres. The morphology of the microspheres is evaluated by SEM, and the size distribution of microspheres is investigated by zeta sizer.

Morphology analysis of gelatin microspheres

The size and shape of ciprofloxacin-loaded gelatin microspheres were further studied by SEM. As shown in Figure 3, the formulated microspheres were spherical and compact in nature. The particle size of the formulated gelatin microspheres was <50 µ as evidenced by the SEM photograph. As shown in the photograph, the particles were less aggregated and they were readily dispersed in water. In addition, there is heterogeneity in size of the microspheres and agglomerates of microspheres were observed.

Particle size analysis of gelatin microspheres

Figure 4 shows the particle size analysis of gelatin microspheres. The particle size measurement showed that the particle size varied from 5 to 50 µm.

DSC analysis

The DSC analysis was performed to find out the physical nature of ciprofloxacin entrapped in the gelatin microsphere and also to confirm the absence of drug–polymer interaction. The thermogram of ciprofloxacin showed [Figure 5] a peak at about its melting point (100.57°C). The thermogram of plain gelatin showed a peak at 96.6°C. Ciprofloxacin peak was absent in the thermogram of drug-loaded gelatin microspheres, which revealed the amorphous nature of entrapped drug in the formulated microspheres.

Figure 5 confirms that there is no shift in glass transition temperature of gelatin and evidence for no crosslinking of ciprofloxacin and gelatin in the microsphere. The DSC analysis was performed to find out the physical nature of ciprofloxacin entrapped in the gelatin microsphere and also to confirm the absence of drug–polymer interaction. The thermogram of ciprofloxacin showed a peak at about its melting point (150.57°C). The thermogram of plain gelatin showed a peak at 96.6°C. Ciprofloxacin peak was absent in the thermogram of drug-loaded gelatin microspheres, which revealed the amorphous nature of entrapped drug in the formulated microspheres.

Table 2: Physiochemical parameters of drug-loaded gelatin microspheres*

Gelatin	Ciprofloxacin	Yield	% Drug loading
1000 mg	750 mg	1544±64	59%
		Yield in %	Encapsulation
		88.2±3.6	78.6%

*Values are reported in mean±SD (*n*=3). SD: Standard deviation

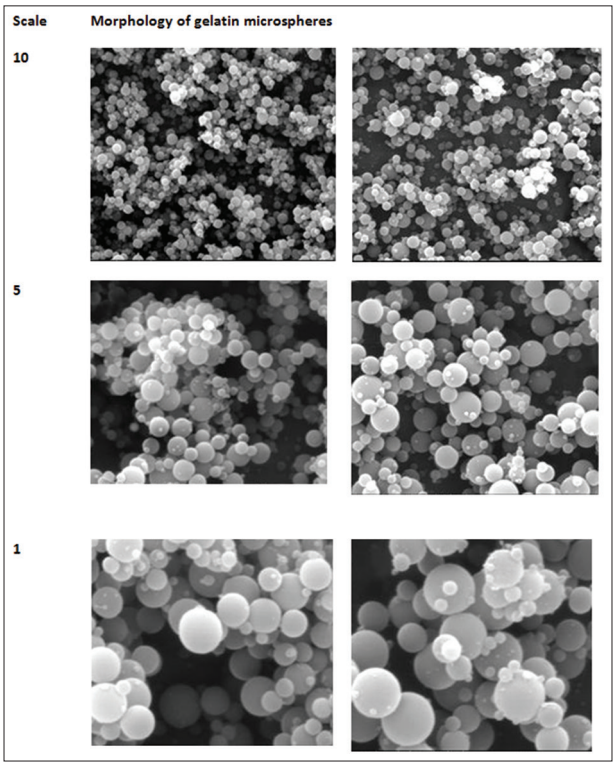

Figure 3: Gelatin microspheres used drug delivery vehicles in the collagen scaffold

Collagen scaffold

Figure 6 shows macroporous collagen scaffold and ciprofloxacin incorporated collagen scaffold. The size of pores in the collagen scaffold is varied from 500 to 600 µm. The pores act as a reservoir of drugs in the scaffold. The ciprofloxacin is physically entrapped into the collagen scaffold.

GMC scaffold

Figure 7 shows the ciprofloxacin-loaded GMC scaffold for infected soft tissue repair. The cross-section of the scaffold confirmed that the microspheres are entangled with the pores of the collagen scaffold. In addition, the interconnectivity pores between fiber attachments to the gelatin microspheres is observed. The size and shape of ciprofloxacin-loaded gelatin microspheres were further studied by SEM. The size of prepared gelatin microspheres is varied from 1000 nm to 10 µm. As shown in Figure 3, the formulated microspheres were spherical and compact in nature. The particles were less aggregated, and they were readily dispersed in water. The percentage of yield of drug loaded gelatin microspheres by water-oil emulsion technique is 88.2% during preparation and recovery and 78.6% drug encapsulated in microspheres. Figure 4 shows the surface morphology of microsphere impregnated collagen scaffold, were collagen scaffold contains pores that entrap physically gelatin microspheres. The SEM observation shows that the pore size of the plain porous collagen scaffold varies from 500 to 600 µm. The pore size of scaffold physically encapsulates gelatin microspheres effectively. The SEM observation shows the microspheres well distributed inside the pores of collagen scaffolds [Figure 8].

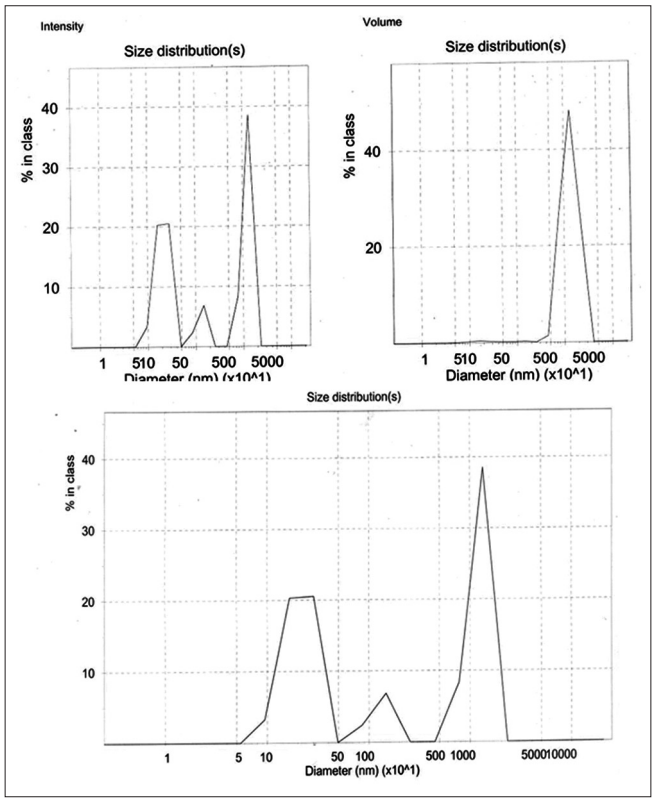

Figure 4: Particle analysis of gelatin microspheres

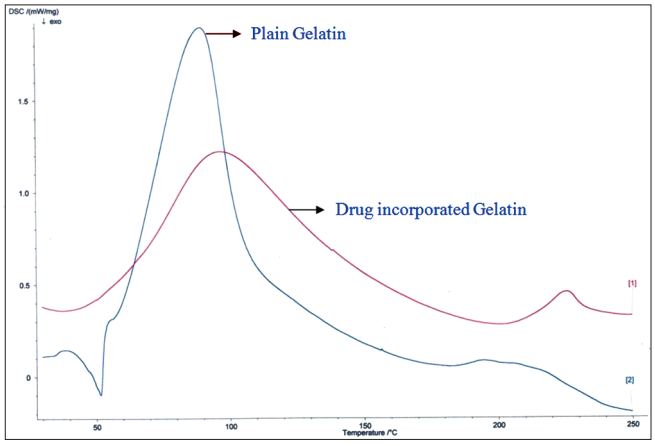

Figure 5: Differential scanning calorimetry thermogram of drug and drug loaded gelatin microspheres

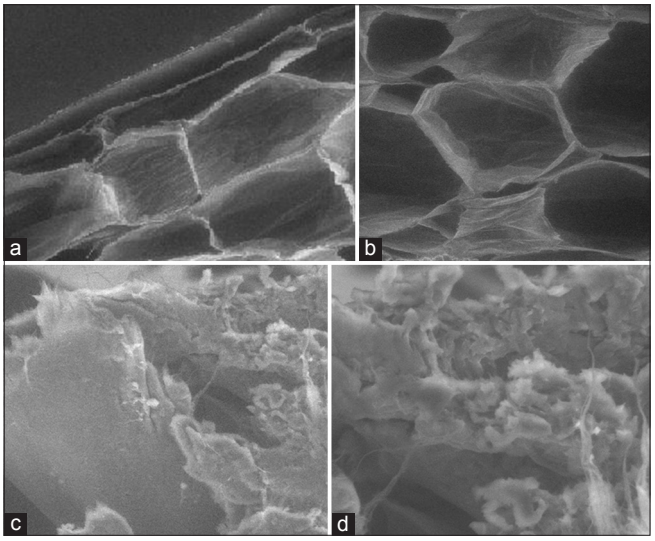

Figure 6: Plain collagen scaffold and ciprofloxacin incorporated collagen scaffold. (a) Cross section of plain collagen scaffold. (b) Plain collagen scaffold. (c) Ciprofloxacin incorporated collagen scaffold. (d) Ciprofloxacin incorporated collagen scaffold

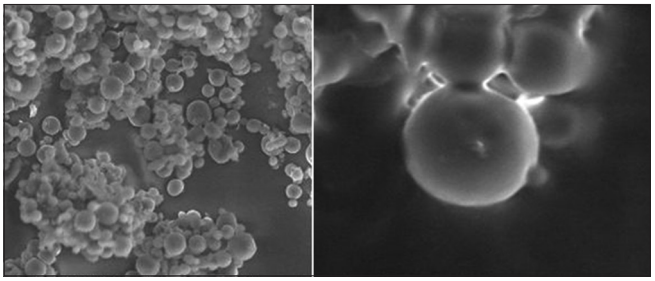

Figure 7: Ciprofloxacin-loaded gelatin microspheres

In vitro drug release profile

The rate of drug release from collagen scaffold [Figures 6 and 7] was then computed for both the fast (1st h) and slow release phases as seen in Figure 7. It can be said that the rate of drug release is proportional to the GA content and cross-linking time for both the fast and slow release phases. Higher the degree of cross-linking slower the release rate and *vice versa*. These results clearly indicate that the release of therapeutic agents from gelatin microspheres can be controlled by varying these parameters. Gelatin microspheres are known to swell in aqueous environments due to hydration. As a new polymeric structure is formed by introducing bridges between polymeric chains during the cross-linking procedure, the extent of the swelling process depends on the degree of cross-linking. Therefore, the denser the cross-linking bridges between the gelatin molecules, the more packed is the structure. Such a structure can be characterized by lower and slower penetration of the solvent through the chain structure of the polymer, suggesting that the swelling ratio and hence the release characteristics of the microsphere can be controlled by varying the content of the cross-linking agent used during the manufacturing process. The complete release of drug was seen in 2 days. This may be due to faster diffusion of dissolution medium or migration. The release rate was initially faster and then became slower as time progress. The initial burst release can be justified using drug that is bound to the surface of the microspheres [Figure 9].

In vitro antimicrobial activity of collagen scaffold

In vitro antimicrobial activity of Gelatin Microspheres, Impregnated Collagen Scaffold shows the zone of inhibition around ciprofloxacin-incorporated collagen scaffold showed a bacterial free zone of 38 ± 2 mm. The reason for the wide zone of inhibition is due to the burst and sustained release of the antimicrobial agent into the microbial culture.

An ideal drug delivery system is one that delivers sufficient quantity of drug at the site of action and prevents further bacterial proliferation at the wound site. The activity of the released drug from the sponge shows a clear zone of inhibition controlling the growth of both cultures inoculated with two different cultures. Drug incorporated collagen scaffold showed a bacterial free zone of 38 ± 2 mm around the gelatin microspheres incorporated collagen scaffold against *Staphylococcus* and *Pseudomonas*. The initial burst release and the sustained release of GMC dressing are responsible for effective antimicrobial activity on the infected wounds. In this work, ciprofloxacin-loaded GMC scaffold provided not only controlled the release of ciprofloxacin but protect the collagen scaffold from microbial degradation. The used drug ciprofloxacin has broad spectrum antimicrobial activity, and the bactericidal activity generated by fluoroquinolones is caused by their inhibition of bacterial DNA gyrase and topoisomerase IV enzymes. DNA gyrase is essential for the replication, transcription, and repair of bacterial DNA

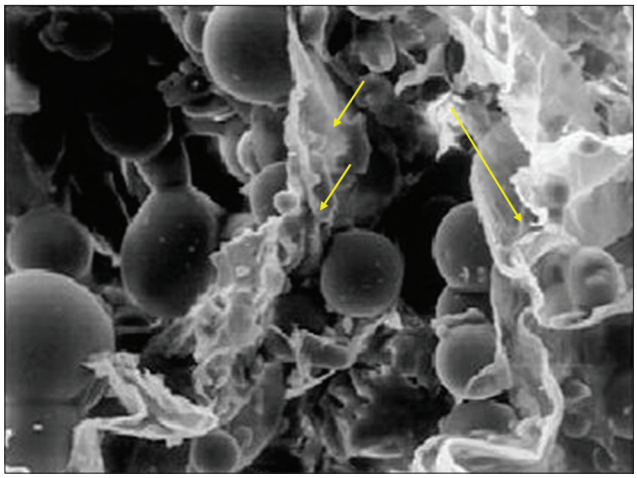

Figure 8: Microspheres impregnated collagen scaffold

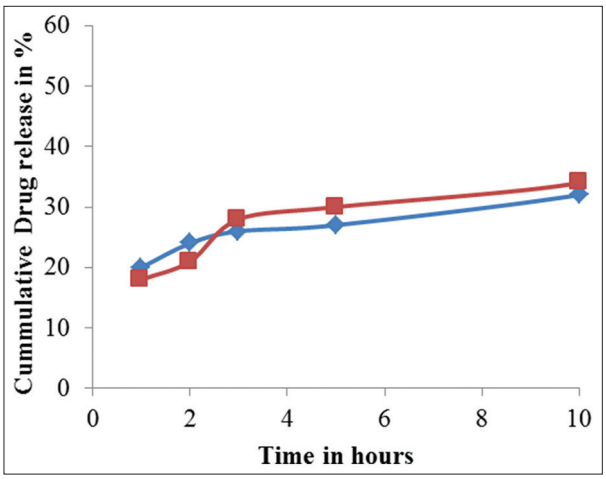

Figure 9: Burst release of ciprofloxacin from the scaffold

and topoisomerase IV is involved in the partitioning of chromosomal DNA during cell wall division. By inhibiting those enzymes, fluoroquinolones keep cellular bacterial DNA in a supercoiled state, thereby preventing bacterial replication.[8]

In vivo evaluation of collagen dressings

Wound contraction in Albino Wister rats

In wound healing process, wound contraction is mediated by specialized fibroblasts (myofibroblasts) found within granulation tissue. These cells are known to contract collagen gel, which was newly synthesized in the site of healing. After tissue injury, revascularization of the wound bed and redevelopment of the extra-cellular matrix is achieved through cell proliferation and the production of granulation tissue. Wound contraction is also an element of the proliferative phase of wound healing, which occurs through the centripetal movement of the tissues surrounding the wound. Figures 10 and 11 increased wound contraction in GMC may be attributed to the enhanced activity of fibroblasts. The slow rate of wound contraction in rats with

collagen scaffold, and the open wound may be attributed to the presence of microorganisms and their metabolites, which affects and inhibits wound contraction and impairs healing. In GMC group, the faster healing rate could be due to the constant and slow release of antibiotic from the dressing. Further, the presence of gelatin also plays an important role in wound healing [Figure 12].

The porous nature of collagen scaffold is efficiently large to absorb and deliver drug and the presence of gelatin microspheres which absorbs water and maintains moist surface at the wound. Wound contraction is mediated by specialized fibroblasts (myofibroblasts) found within granulation tissue. These cells are known to contract collagen gel, which was newly synthesized in the site of healing. After tissue injury, revascularization of the wound bed and redevelopment of the extra-cellular matrix is achieved through cell proliferation and the production of granulation tissue. Wound contraction is also an element of the proliferative phase of wound healing, which occurs through the centripetal movement of the tissues surrounding the wound. Figure 12 shows increased wound contraction in GMC (GMC scaffold) group due to the enhanced activity of fibroblasts. The slow rate of wound contraction in rats with collagen scaffold, and the open wound may be attributed to the presence of microorganisms and their metabolites, which affects and inhibits wound contraction and impairs healing. In GMC group, the faster healing rate could be due to the constant and slow release of antibiotic from the dressing. Further, the presence of gelatin also plays an important role in dermal regeneration [Figure 13].

Histological analysis of granulation tissue from infected wound

The H and E staining of granulated tissue confirms healing progress in the Wister rats. In open wound group, the bacterial colonies were seen and no formation of dermis and epidermis at the wound site. In the case of plain collagen scaffold and ciprofloxacin collagen scaffold, the significant formation of dermis and epidermis were over served. When compared to the groups treated with ciprofloxacin-loaded GMC scaffold, the well-formed epidermis and dermis were observed. In the histological studies, ciprofloxacin-loaded GMC scaffold treated group shows epithelialization with the moderate extracellular matrix on the day 12 whereas, in the control group, incomplete epithelialization with less extracellular matrix synthesis and persistence of inflammatory exudates in the upper dermis with the loss of epidermis were observed up to day 12 [Figure 14].

Masson's trichrome staining of granulation tissue

Figure 13 shows collagen content in the granulated tissue by Masson's trichrome staining of granulated tissue collected from the wound site. When comparing the control group (open wound, Plain collagen, ciprofloxacin incorporated

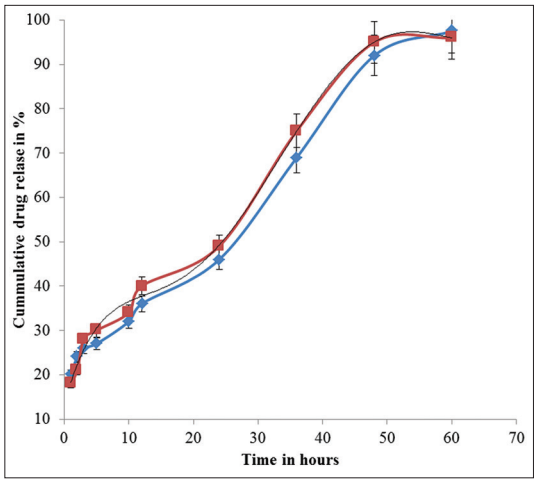

Figure 10: Sustained release from the microspheres impregnated collagen scaffold

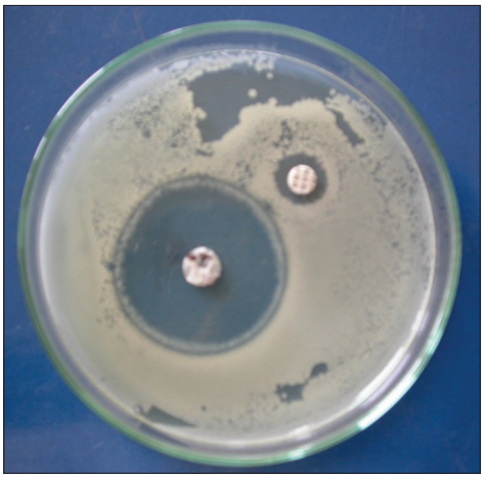

Figure 11: Zone of inhibition produced by microspheres impregnated collagen scaffold

collagen scaffold) with a treated group (ciprofloxacin-loaded gelatin microsphere impregnated collagen scaffold), the collagen bundles are formed in the treated group and well-formed dermis and epidermis has been observed in the treated group. Similarly, Masson's Trichrome staining of granulated tissue confirms that well organized formed collagen bundles observed in the group treated by GMC. Moreover, the dermis and epidermis of the GMC group are effectively regenerated and complete wound closure has been observed when comparing with other groups. Furthermore, GMC scaffold effectively controls bacterial load at the wound site and completely combats wound pathogen at 12th day of wound healing when comparing with other groups [Figure 15].

Microbiological examination of granulation tissue

Table 3 confirms the rate of the infection completely eradicated in the treated group than that of control groups. In addition, it is observed that there is no presence of wound pathogens at the wound site of the group treated by microspheres incorporated collagen scaffold. In summary,

Table 3: Infection control at wound site

Time in days	Control gauze dressings	Plain collagen scaffold	Ciprofloxacin incorporated collagen scaffold	Microspheres incorporated collagen scaffolds
4	1.00×10^{9}	1.00×10^{8}	1.00×10^{7}	1.00×10^{5}
8	1.00×10^{8}	1.00×10^{7}	1.00×10^{5}	1.00×10^{3}
12	1.00×10^{6}	1.00×10^{6}	1.00×10^{3}	No

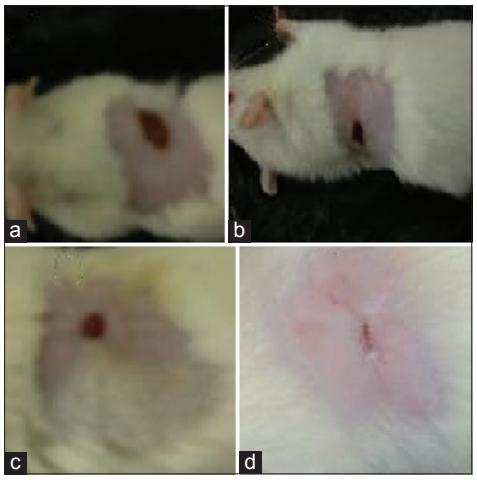

Figure 12: Wound contraction at 12th day: (a) Open wound group, (b) plain collagen scaffold, (c) ciprofloxacin incorporated collagen scaffold, (d) ciprofloxacin-loaded gelatin microspheres impregnated collagen scaffold

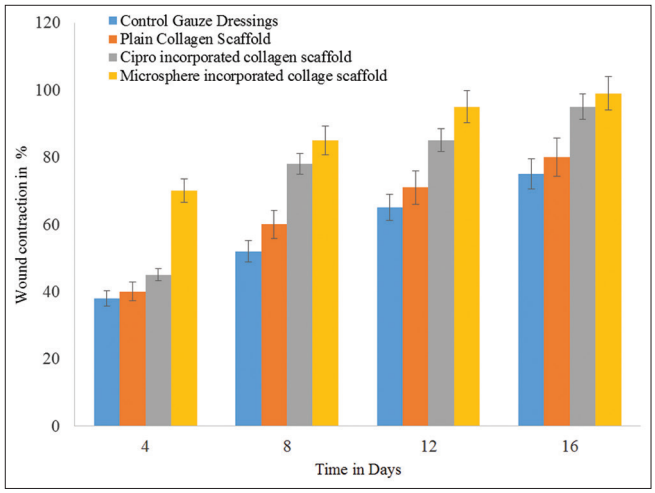

Figure 13: Wound contraction in albino Wister rats

currently developed microspheres impregnated collagen scaffold had been designed to deliver drug over the wound surface in a uniform and sustained fashion. The biomaterials used to develop this system are of natural origin, which minimizes possible toxicity due to the core materials used. Apart from that, collagen and gelatin by virtue possess active wound healing property. Collagen scaffold used as delivery device adds up more advantage to the present system since it minimizes dressing frequency makes examination easier and assessment of wound site with added esthetic value.[29-31]

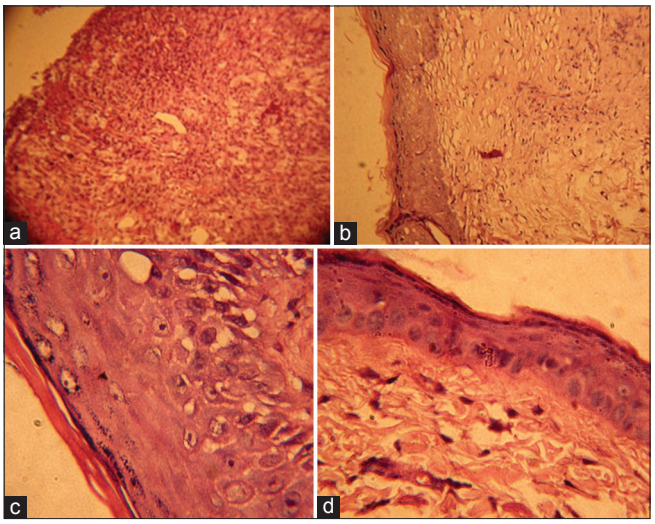

Figure 14: H and E staining of granulated tissue collected at 12th day of wound healing. (a) Open wound. (b) Plain collagen scaffold. (c) Ciprofloxacin incorporated collagen scaffold. (d) Gelatin microspheres impregnated collagen scaffold

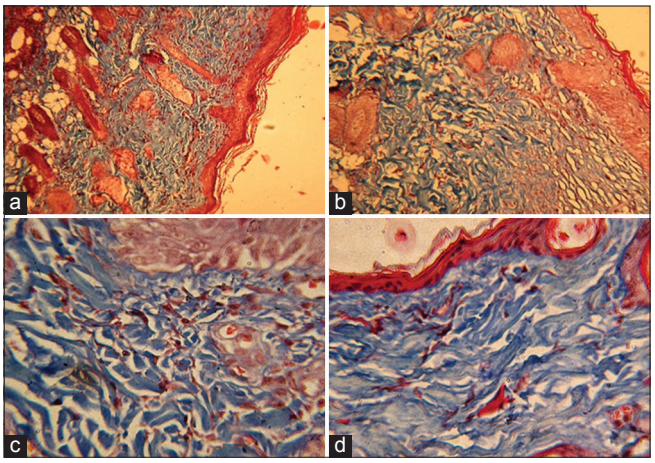

Figure 15: Masson's trichrome staining of granulated tissue. (a) Open wound. (b) Plain collagen scaffold. (c) Ciprofloxacin incorporated collagen scaffold. (d) Gelatin microspheres impregnated collagen scaffold

CONCLUSION

Recent advances in collagen scaffold technology have led to the emergence of topical products that do more than just cover and conceal, but address specific issues in highly infected wounds and facilitate the regeneration process of dermis and epidermis. The present investigation deals the scope for

applying the antimicrobial agent loaded gelatin microspheres into the collagen scaffold for combating infection in the wound site as well as to provide a controlled release of ciprofloxacin into the wound site effectively and resulting in complete wound closure. Thus, the developed wound care system is likely to provide a controlled environment for healing as well as deliver the drug at a required concentration to control infection and further release of ciprofloxacin into the wound site.

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