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FABRICATION AND ASSESSMENT OF CIPROFLOXACIN MICROSPHERES LOADED COLLAGEN BASED 3D SCAFFOLDS

SRINIVAS MURTHY BR[1*], PRASANNA RAJU Y[1], VISHAKHA K[1], JAMAL BASHA D[2], REKHA DEVI A[3], SHANMUGAM V[4] AND LAKSHMAYYA B[5]

1: Pharmaceutics Division, Sri Padmavathi School of Pharmacy, Tiruchanoor, Tirupati, IN-517503

2: Department of Pharmacognosy, Amity Institute of Pharmacy, Amity University, Gwalior, Madhya Pradesh, India

3: Department of Pharmaceutics, Seven Hills college of Pharmacy, Tirupati, India

4: Pharmaceutics Division, Sri Vidyanikethan College of Pharmacy, TIrupati, India

5: Department of Pharmacognosy, Vydehi Institute of Pharmacy, Whitefield, Bangalore, IN-560066

[*]**Corresponding Author: Dr. Srinivas Murthy BR; E Mail:** seenu46@gmail.com

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ABSTRACT

Objective: The research was focused to fabricate and assess ciprofloxacin loaded microspheres and subsequent impregnation of microspheres into collagen based 3-D scaffolds for controlled delivery.

Methods: Ciprofloxacin chitosan microspheres were developed by ionic precipitation and chemical cross-linking method later characterized for particle size, surface morphology, drug entrapment efficiency and *in-vitro* drug release. Adjacently, collagen (type-I) was extracted from bovine tendons and characterized for hydroxyproline content, circular dichromism and electrophoresis methods. The optimized microspheres were impregnated in 1% collagen and fabricated into a three dimensional scaffold by lyophilization. Similarly the microspheres were also impregnated into chitosan and PVA scaffold of 1% each for comparison of drug release patterns. The scaffolds were characterized for morphology, pore size distribution,

swelling, chemical incompatibility, thermal behavior, *in-vitro* drug release and anti-bacterial (collagen scaffolds) efficiency.

Results: The average particle size of microspheres were found to be 229 μm with porous surface texture, entrapment efficiency of 67.66% and controlled drug release (98.04%, 14h). The collagen extracted was found to be of good purity. All the scaffolds showed good morphology with even pore size distribution, acceptable swelling index and thermal stability. First-order release with initial burst release of drug from collagen scaffold was noticed and followed by controlled release over several hours.

Conclusion: The collagen scaffolds containing microspheres exhibited its supremacy over other scaffolds in all parameters and effective against selected bacterial strains. Thus, collagen based scaffold loaded with microspheres containing antibacterial agent serves as a promising biomaterial in biomedical applications.

Keywords: Ciprofloxacin, collagen, ionic-gelation, lyophilization, microspheres, scaffolds

1. INTRODUCTION

Biomaterials are the substances that have been engineered to interact with biological systems to treat, augment or replace any tissue or organ of the body. Amongst them, collagen is widely used because of its excellent biocompatibility and biodegradability. Biomaterials play a vital role in tissue engineering which is an interdisciplinary field and is well associated with the principles and methods of engineering and life-sciences **[1]**. Tissue engineering has become a promised alternate approach to treat the lost/damaged tissue or an organ with the help of biomaterials by surmounting the limitations of current pharmacotherapy and surgery **[2-4]**. Employing tissue engineering, amyotrophic lateral sclerosis was treated by encapsulating ciliary neurotrophic factor (CNTF) secreting neural cells **[5]** and in ophthalmological engineering so as to develop cornea, retina, lens tissue and dental pulp from dental fibroblast and synthetic dental matrix **[6-14]**.

In recent past, tissue engineering principles associated clinical applications are fortified by adjoined biodegradable polymers. Appropriate polymers are identified to fabricate and develop the porous three dimensional scaffolds for biomedical applications in recent times **[15-19]**. The biodegradable, synthetic or semi-synthetic polymers such as PGA, PLGA, PLA and collagen are evidently superior to apply in tissue engineering field. Applicability and suitability of above polymers were confluence by the novel techniques such as gas foaming, fiber extrusion-bonding, 3-D printing, phase separation, emulsion freeze drying, porogen

leaching and solvent casting-drying **[20-22]**. Unlike other polymers, collagen is the most abundant protein in human body accounting for 30% of total proteins. Owing to excellent biocompatibility, biodegradability, and weak antigenicity, the collagen remains as a promising biomaterial in tissue engineering especially for scaffolding. Collagen excelled its role in burn/ wound dressings, osteogenics, bone filling/ substitution process, skin replacement, artificial blood vessels and valves **[23-24]**.

Ciprofloxacin, a broad-spectrum flouroquinolone antibiotic has high penetration ability with 60% of oral bioavailability, biological half-life of 3-6 hr, pKa of 6.09, and being excreted 40-60% unchanged in urine. Ciprofloxacin is widely used in the treatment of several pathological diseases and infections including in multi drug resistant cases/ conditions. The oral administration of ciprofloxacin is common, but cause severe gastric complications including abdominal cramps and other adverse effects too **[25]**. Microspheres particularly made up of natural biodegradable polymers like chitosan are novel drug delivery carriers that enhance the efficacy of drug moieties with desired drug release. With this background, the present research was aimed at designing ciprofloxacin microspheres and subsequent incorporation of microspheres into fabricated collagen 3-D scaffolds for extended release. The comparative evaluation of 3-D scaffolds with chitosan and poly vinyl alcohol (PVA) was also aimed here to establish the efficacy of the formulation.

2. MATERIALS AND METHODS

Ciprofloxacin was the gratis of M/s Medrich Pharmaceuticals, Bengaluru. Chitosan and Poly vinyl alcohol (PVA), Triton X-100 were procured from S.D. Fine Chem Ltd., Mumbai. All other chemicals and solvents used were of analytical grade.

2.1.1. Fourier Transform Infrared (FT-IR) analysis

FT-IR spectra of ciprofloxacin, chitosan and their physical mixture were recorded on a Thermo-IR 200 spectrophotometer. Potassium bromide pellet method was employed and background spectrum was collected under identical conditions. Each spectrum was derived from 16 single average scans collected in the range of 4000-400 cm^{-1} at the spectral resolution of 2 cm^{-1} **[26]**.

2.1.2 Preparation of ciprofloxacin microspheres

Ionic precipitation-chemical cross-linking (IPCC) method was employed to formulate ciprofloxacin-chitosan microspheres. In this method, five formulations with varying drug- polymer ratio were prepared as given in **Table 1** and were coded as F1 to F5. Initially specified

quantity of chitosan was dissolved in 1% dilute acetic acid and 500 mg of ciprofloxacin was added drop wise to the chitosan solution under continuous stirring. 2 ml of span 20 was added to the resulting mixture with constant stirring. 2% w/v of sodium sulphate was also added and stirring was continued for 30 min. Then 0.5ml of saturated glutyraldehyde (GTA, crosslinking agent) was added and stirring was continued for another 30 min. The resulting mixture was centrifuged at 5000 rpm for 15 min, microspheres thus obtained was separated, washed, dried at room temperature for 24 hrs and stored in vacuum desiccator for further use **[27, 28]**.

Characterization of microspheres

2.2.1: Particle size analysis and morphology

Particle size of ciprofloxacin loaded microspheres was analyzed by particle size analyzer (Shimadzu, SALD-3101) and surface morphology was studied by Scanning Electron Microscopy (Hitachi, SEM-SU 8020) at an accelerating voltage of 10 kV.

2.2.2: Entrapment efficiency and swelling index

The amount of ciprofloxacin entrapped in microspheres was determined by dissolving weighed quantity of microspheres in distilled water. The solution was equilibrated for 2 hr and filtered through 0.45 µm nylon filter. The resulting samples were analyzed for free drug concentration and entrapment efficiencies. Swelling behavior of the cross-linked microspheres was estimated by placing weighed quantity of microspheres in 50 ml reservoirs containing phosphate buffer (pH 7.4) and were agitated at 100 rpm at 37ºC for 1 hr. The differential of weights between initial and swollen samples was considered to calculate the degree of swelling **[29]**.

$$\text{Swelling index (\%)} = W_0\text{-}W_1/W_0 \text{ X } 100 \quad (1)$$

2.2.3: *In-vitro* drug release

In-vitro drug release studies for microsphere formulations (F1-F5) were carried out using phosphate buffer (pH 6.8) solution as dissolution medium at 37±0.2° C. The ciprofloxacin loaded microspheres were added to 50 ml of dissolution media in stopper bottles. The bottles were fixed in the Remi orbital shaker and speed was adjusted to 50 rpm. Aliquot of 5 ml samples were withdrawn at predetermined time intervals by following sink condition. The samples were filtered and analyzed by UV spectroscopy at 272 nm. The cumulative percent of ciprofloxacin release from cross-linked microspheres was calculated as function of time **[30]**.

Table 1: Formulation of ciprofloxacin-chitosan microspheres

Ingredients	F1	F2	F3	F4	F5

Ciprofloxacin	**500 mg**	**500 mg**	**500 mg**	**500 mg**	**500 mg**
Chitosan	**500 mg**	**625 mg**	**750 mg**	**875 mg**	**1000 mg**
Span 20	**2 ml**	**2 ml**	**2 ml**	**2 ml**	**2 ml**
Sodium sulphate (2%)	**10 ml**	**10 ml**	**10 ml**	**10 ml**	**10 ml**
GTA	**0.5 ml**	**0.5 ml**	**0.5 ml**	**0.5 ml**	**0.5 ml**

2.3: Extraction of type-I collagen

Extraction of type-I collagen from bovine tendons was done according to the patented method of Bioproducts Lab, CLRI, Chennai. In this method, bovine tendons were collected from the local slaughterhouse. Adhered non-collagenous tissues and fat were removed by salting technique. The tissue was cut, minced and treated with Triton X-100. They were further subjected to alkali treatment for swelling and removal of left-over fat and non collagenous tissues. Non-helical regions were also removed by proteolytic enzymatic treatment of the tissue. Telopeptide, an enzyme that has immunogenic response was deactivated by treating with alkali and washed thoroughly. Finally pure collagen of type-I sample was collected.

2.4: Characterization of type-I collagen

Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to determine the position and distribution of amino acid bands (α, β and γ) in type-I collagen. Previously reported protocol system was used to run the electrophoretogram. Slab gel (8×7 cm) was cast using supporting, separating and stacking gel system **[31, 32]**.

Determination of hydroxyproline content in type-I collagen

In order to establish the type, structure and purity of collagen, hydroxyproline content was estimated. The collagen pellet (freeze dried) was taken into hydrolysis tube (Wilmed LG-10803-100) to which 5 ml of 6N HCl was added. The hydrolysis tube was sealed and kept in hot air oven at 110°C. After 16 hours the hydrolysis tube was broken and the content was transferred into crucible. The crucible was kept under heating to evaporate HCl and the residue was evaporated twice with 5 ml of distilled water. Finally the residue was dissolved in a 5 ml of distilled water, filtered and then absorbance was read. Total hydroxyproline content was calculated using equation **[32, 33]**.

$$\text{Total Hydroxyproline} = \text{Absorbance} \times 7.36 \quad (2)$$

Circular Dichroism studies of type-I collagen

Circular Dichroism (CD) is used to analyze triple helix conformation of collagen molecule. The collagen samples were prepared in 0.25 M acetic acid and equilibrated overnight. The samples were spun to remove undissolved protein. CD was examined in Jasco J-500A spectropolarimeter containing 1 mm length

unit cell. Liquid nitrogen was circulated through the instrument for about 30 minutes to attain and maintain a constant temperature in the system and not to affect the nativity of collagen during experiment. Baseline was adjusted to zero using 0.25M acetic acid as blank **[31, 34]**.

2.5: Fabrication of microspheres impregnated collagen based scaffolds

The collagen scaffolds were fabricated by emulsification followed by freeze-drying process. Initially 1% w/v of collagen was prepared and set aside for complete swelling of collagen fibers with occasional stirring. Ciprofloxacin loaded microspheres (equivalent to 100mg) was added to 100 ml of collagen solution and homogenized for 20-30 min. Entrapped air bubbles was removed by vacuum treatment. The homogenized microspheres containing collagen solution was casted onto moulds (5.02 cm^2/1.2 mm) and lyophilized for 48-72 hr to obtain porous ciprofloxacin microspheres loaded collagen scaffolds (CMCS). Same procedure was followed to fabricate microspheres loaded scaffolds of chitosan (1%) and PVA (1%) **[33]**.

2.6: Characterization of scaffolds

2.6.1: Physical characterization

The scaffolds were subjected for physical characterization like colour, shape, size and transparency.

2.6.2: Morphology, pore size distribution and drug content

The morphology and pore size distribution of collagen was determined by using Scanning Electron Microscopy. The scaffolds were randomly cut into four equal sized (2.02 cm^2) countours and homogeinity (drug content) of microspheres with in the scaffolds was assessed by dissolving them in methanol and analyzed for amount of ciprofloxacin spectrophotometrically.

2.6.3: Thermal behavior

Thermal decomposition of proteins and incompatibility issues between the ingredients of scaffolds were examined. Differential Scanning Colorimetry (DSC) (Perkin Elmer's DSC-4000) and Thermo gravimetric analysis (TGA) (Shimadzu's TGA-50 model) techniques were employed to understand the thermal behavior of scaffolds **[34]**.

2.6.4: In-vitro drug release from scaffolds

Drug release from scaffolds was assessed by placing the respective scaffold of 2 cm^2 size in 50 ml of pH 7.4 phosphate buffer solutions and on orbit shaker. 1 ml of sample was withdrawn at every 1 hr by following the sink condition. The samples were filtered and assayed. Study was conducted for three independent observations. Average of percent drug release was calculated and drug release data were fitted into various kinetic models in order to establish the order of drug release **[35]**.

2.6.5: In-vitro antibacterial activity

The antibacterial efficiency of collagen scaffolds was determined by measuring Minimum Inhibitory Concentration (MIC). Selected bacterial strains viz., *Staphylococcus aureus*, ATCC29213 (Gram +ve) and *Escherichia coli* ATCC25922 (Gram -ve) were cultured by using 96-well micro plate method in Mueller-Hinton broth. The bacterial suspension was seeded to the Mueller-Hinton agar medium and incubated at 37°C for 24 hr. Ciprofloxacin microspheres loaded collagen scaffolds and plain scaffolds (without drug) were placed on agar medium and incubated for 24-48h. The antibacterial efficacy was assessed by measuring zones of inhibition under strict aseptic conditions **[36]**.

3. RESULTS

3.1: Preformulation studies- FTIR

FT-IR spectra of ciprofloxacin, chitosan and their physical mixture were shown in **Figure 1**. Spectra demonstrated that, a prominent characteristic peak was found at 3329 cm^{-1} which was assigned to stretching vibration of NH group. The carbonyl C=O stretching was observed at 1702 cm^{-1}. A strong absorption peak at 1027 cm^{-1} was assigned to C-F group. The NH group was further confirmed with the bending vibration of C-N peak around 1267 cm^{-1}. The melting point of the drug was found to be 331.2° C.

3.2: Physicochemical characterization of microspheres

3.2.1: Particle size and morphology

The average particle size of formulations was in the range of 229.2 µm as shown in **Figure 2(a)**. The microspheres were found to be free flowing with slight yellowish orange colour due to degree of cross-linking between chitosan and glutyraldehyde (GTA). The SEM photograph of F3 as shown in **Figure 2(b)** elucidated that the microspheres possessed slightly rough and porous surfaces.

3.2.2: Entrapment efficiency (EE) and swelling index

Entrapment efficiencies of formulations were ranged from 43.20% to 62.66 %, where F3 formulation exhibited 62.66% of highest EE with swelling index of 54.22%.

3.2.3: In-vitro drug release

The drug release patterns with function of time were depicted in **Figure 3(a)**. Amongst all, F3 formulation exhibited highest drug release (94.08%) at the end of 14^{th} hr.

3.3: Characterization of type-I collagen Electrophoresis (SDS–PAGE), Hydroxyproline content and circular dichroism

In order to confirm the type of collagen obtained from the extraction process, SDS-PAGE electrophoresis was conducted. The information was used to

predict the monomeric, dimeric, trimeric and higher polymeric forms (α, β and γ) of collagen present in the sample of extracted collagen. The hydroxyproline content was found to be 84.06% which confirmed the purity of type-I collagen. Circular dichroism spectrum of collagen is shown in **Figure 3(b)**.

FT-IR study of collagen

FT-IR spectrum of type-I collagen was depicted in **Figure 4(a)**. The peak around 1632 cm^{-1} arises from the C=O stretching vibration of amide groups in the protein A single peak at 3799 cm^{-1} was due to secondary NH group of amide. The amide was further confirmed with the bending vibration of C-N peak around 1130 cm^{-1} and NH bending vibration at 1566 cm^{-1}.

3.4: Characterization of scaffolds

3.4.1: Physical examination

The morphological character of collagen, chitosan and poly vinyl alcohol (PVA) scaffolds exhibited with white to slightly yellowish colour with consistently porous with 5.02 cm circular dimension. Among them pure ciprofloxacin loaded scaffold was white in colour whereas microspheres loaded collagen scaffolds possessed slight yellow colour. The pure ciprofloxacin scaffolds of chitosan and PVA were transparent and microspheres loaded scaffolds were opaque due to the distribution of microspheres.

3.4.2: Morphology and pore size distribution

The cross sectional SEM image of collagen scaffold was depicted in **Figure 4(b)** and it was demonstrated that ciprofloxacin microspheres were distributed uniformly in scaffold and exhibited interconnected 3-D porous structure with pore size of 100- 200 µm.

3.4.3: Swelling indices and Drug content

The swelling indices of scaffolds were shown in **Table 2**. The swelling indices of three scaffolds were found to be 265.63%, 192.13% and 189.37 % for collagen, chitosan and PVA scaffolds. Similarly the drug content for collagen, chitosan and PVA scaffolds found to be 89.64%, 80.83% and 76.10% respectively.

3.4.4: Thermal study

Figure 5(a) elucidated that DSC thermograms of collagen scaffold showed a primary transition temperature at 110-120°C and that of chitosan and PVA scaffolds at 90-110°C. In addition, chitosan and PVA scaffolds showed a secondary transition at 210-220°C. An increase in the denaturation temperature due to the extensive cross-linking with GTA was observed. **Figure 5(b)** demonstrated TGA curves of collagen, chitosan and PVA scaffolds where thermal degradation of scaffolds showed a two step temperature transition as well as corresponding weight loss which was accounted for the function

of cross-linking efficiency. The initial dehydration of bound water moieties at 50-90°C, polymer denaturation was observed at 250-400ºC and the final transition was observed at above 400-700ºC.

3.4.5: Drug release from scaffolds

As shown in **Figure 6**, the drug release from the collagen scaffold was immediate than chitosan and PVA scaffolds. 98.75% of ciprofloxacin was released from collagen scaffold on 12th hour with an initial burst release and found be highest when compared to that of other scaffolds.

3.4.6: Antibacterial activity of the collagen scaffold

The scaffolds displayed clear zones of inhibition of 14 mm and 10 mm against *S. aureus* and *E. coli* (following diffusion mechanism) against *S. aureus* and *E. coli* respectively as shown in **Figure 7**.

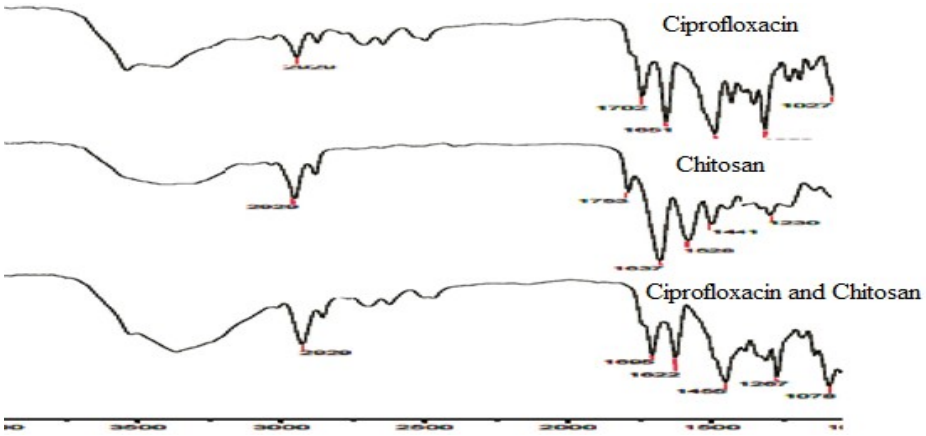
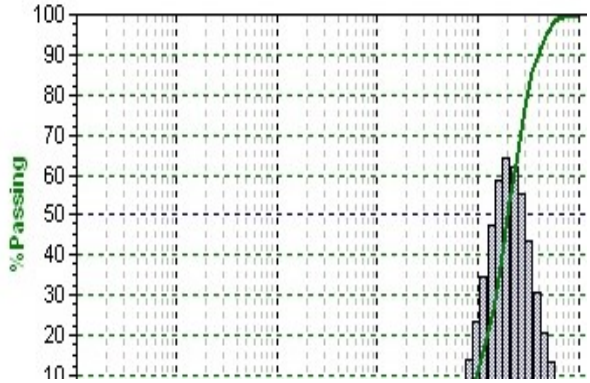


Figure 1: FTIR of ciprofloxacin, chitosan and their physical mixture



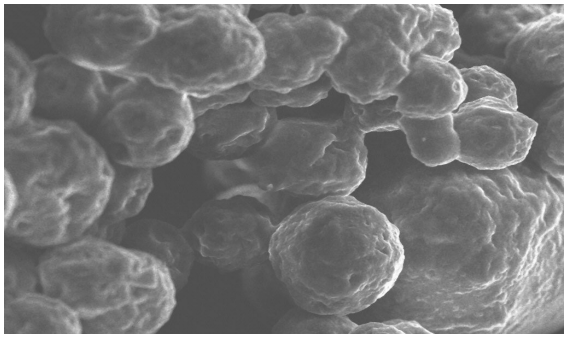
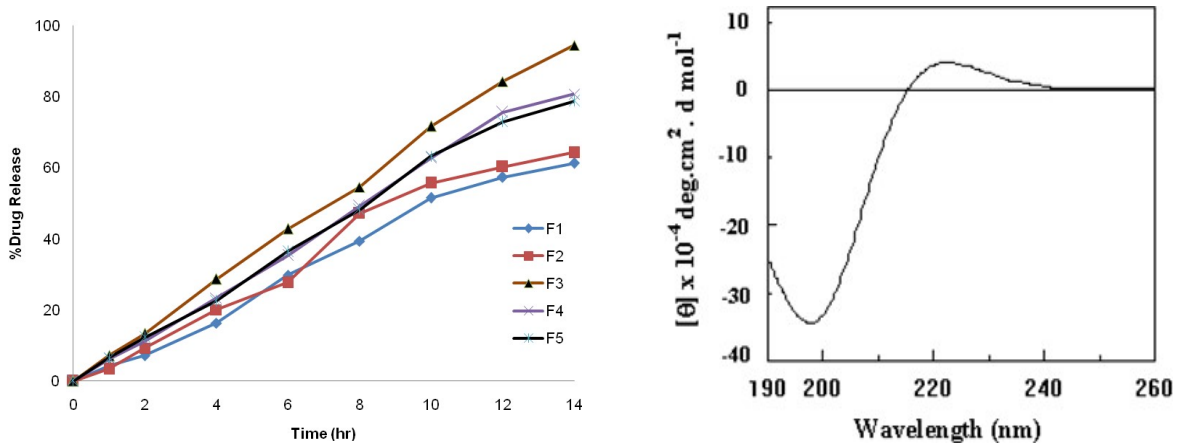


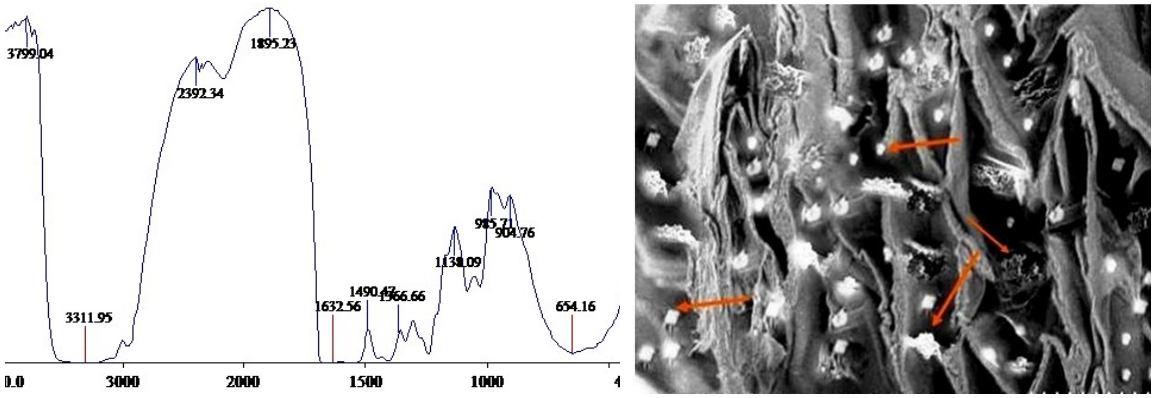
Figure 2(a): Particle size distribution of microspheres

Figure 2(b): SEM image of ciprofloxacin-chitosan microspheres



(a) **(b)**

Figure 3: (a) Drug release from microspheres (b) Circular dichroism of collagen



(a) **(b)**

Figure 4: (a) FTIR of collagen (b) SEM of chitosan microspheres loaded collagen scaffold

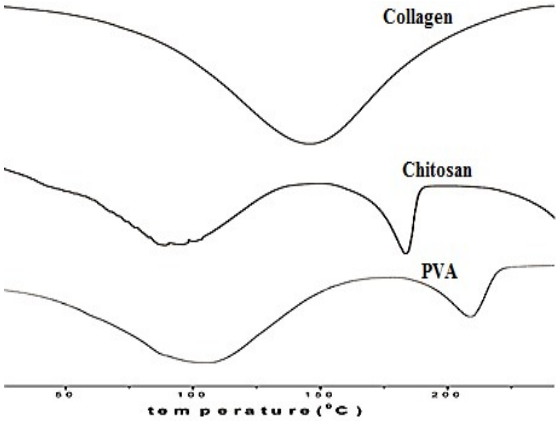


Figure 5(a) DSC thermograms of scaffolds

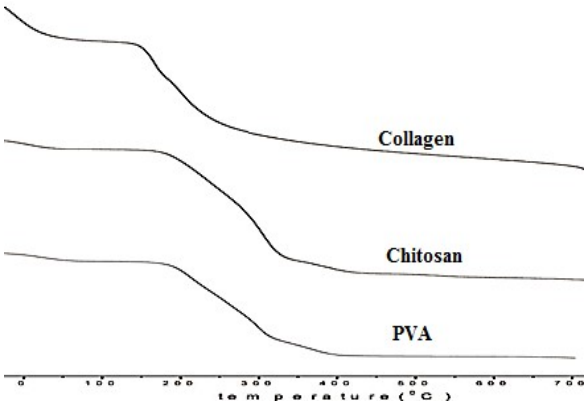


Figure 5(b). TGA curves of scaffolds

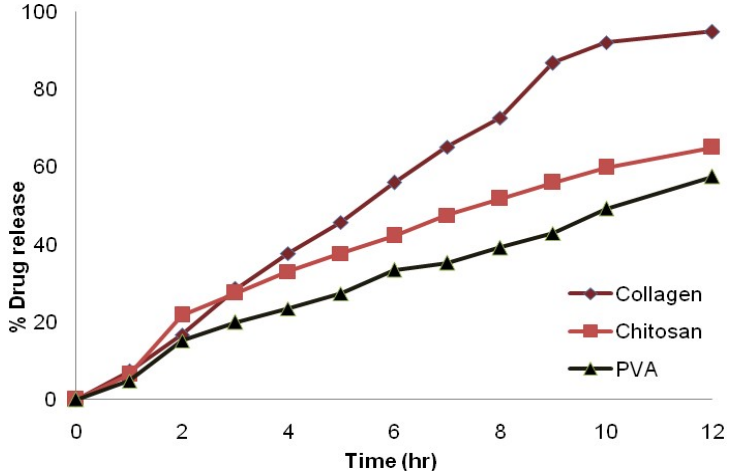


Figure 6: Dissolution profiles of ciprofloxacin from scaffolds

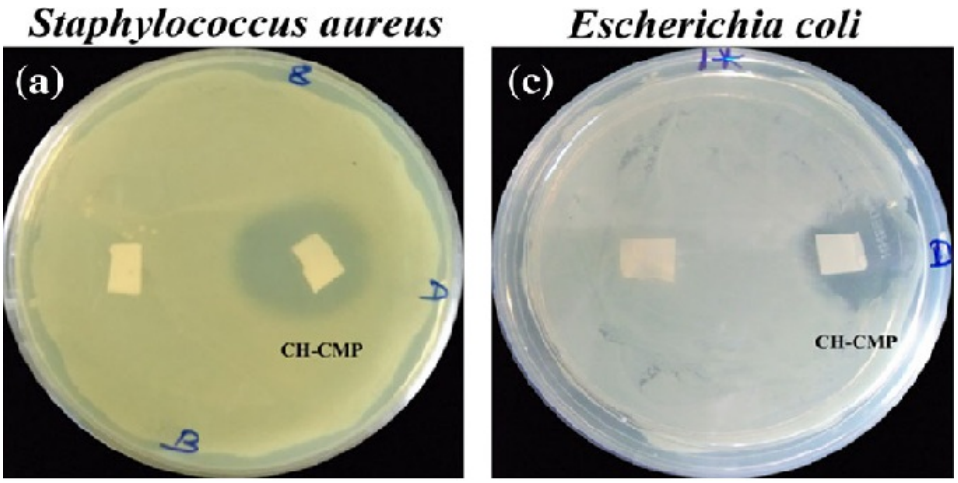


Figure 7: Antimicrobial activity of the microspheres loaded scaffold and plain scaffolds against *S. aureus* and *E. coli*

4. DISCUSSION

The collagen based 3-D scaffolds were fabricated by emulsification- freeze drying technique, in which ciprofloxacin microspheres processed by IPCC method were impregnated. This approach stands

out to be simple and scalable method and has been widely preferred in the past few years. In this study, five formulations of ciprofloxacin-chitosan microspheres were prepared, out of which, formulation F3 was promised and selected to impregnate into 3-D scaffolds.

Evaluation of various physicochemical characteristics of microspheres showed that, drug and polymer were chemically compatible without any irreversible physicochemical interactions as shown in **Figure 1**. The IPCC method employed was proved to be superior in producing sub-micron ranged particles (**Figure 2(a)**) with free flowing nature. Potentials of chemical cross-linking between GTA and chitosan were evident with the formation of coloured, slightly rough and porous surfaced microspheres as shown **Figure 2(b)**. Some of the microspheres were very slightly aggregated which could be due to experimental cross-linking conditions such as time and amount of GTA. Employed conditions, particularly applied thermal condition facilitated to have highest entrapment efficiency, optimum swelling index and highest percent of drug release of F3 formulation. Based on the characteristics, F3 was selected for impregnation into scaffolds.

The type of collagen extracted was confirmed by SDS-PAGE electrophoresis. Separation of α, β and γ chains were achieved on the basis of molecular weight of each species of collagen on a separating gel of SDS-PAGE. As per the results (figure not shown), the percentage distribution of the α_1 band was twice and as large as that of the α_2 band. These results were consistent with the fact that helical structure of type-1 collagen was composed of three polypeptide chains α_1, $ß_1$ and α_2. The hydroxyproline content in the collagen samples provides information about type, quality and purity of the collagen. The results of study showed that 84.06 % of hydroxyproline was present in the extracted collagen sample of type-I. It was also noticed in the structure of type-I collagen, Gly – x – y (formal primary sequence of collagen), the Y position was predominantly occupied by hydroxyproline. Triple helix of type-I collagen was confirmed by circular dichroism spectrum (showing π- π^* transition peak at 196.5 nm and positive n- π^* transition at 220 nm). Further, the functional groups evidently present in the extracted collagen were of similar to that of standard type-I collagen.

The ciprofloxacin-chitosan microspheres were distributed uniformly in the scaffolds. This even distribution of micro-porous structures as shown **Figure 6**, in 3-D scaffolds can facilitate to enhance cell intrusion, proliferation and function of tissue engineering. Thus results of the study outreach to biomedical needs. The ability

of a scaffold to preserve water is an important aspect to evaluate its property for skin tissue engineering. Emulsification followed by freeze drying technique produced the dehydrated scaffolds, which in-turn absorbs quantum of water. Water binding ability of the collagen/chitosan scaffolds were due to hydrophilicity of dehydrated 3-D structured scaffolds. Swelling of scaffolds was increased with temperature and thus, scaffolds can cater the skin tissue engineering needs.

There was no incompatibility in the microspheres loaded scaffolds and hence, it was clearly indicated that incorporation of microspheres into the collagen and other scaffolds had not altered any structural properties as well physical integrity of the scaffolds. Owing to high porosity and fibrous like networking structure of collagen, resulted the uniform distribution of microsheres compared to other two scaffolds. Thermal behavior of scaffolds with primary transition (denaturation or melting) demonstrated thermal stability of collagen scaffold. Thermal transition temperature 'T_d', at which the triple helical structure of collagen was converted into random coil structured collagen. Hence, it was termed as helix-coil transition which depends on degree of hydration. Increase in denaturation temperature of chitosan and PVA scaffolds to higher temperature when compared with collagen was due to increased inter and intra fibrillar interaction. As noticed in TGA curves, thermal degradation of scaffolds was initiated and therefore it was considered as a decomposition phase or carbonization of polymer. Dissolution profiles of collagen scaffold exhibited immediate drug release compared to other scaffolds with an initial burst release in First-order manner followed by slow release. Ciprofloxacin was released from pure ciprofloxacin loaded scaffolds immediately compared to that of microspheres impregnated scaffolds. The slow/delayed drug release patterns were attributed due to entrapment of ciprofloxacin in chitosan microspheres and moreover unifrom distribution and good homogenicity of microspheres in scaffolds. The scaffolds exhibited good antibacterial activity against *S. aureus* and *E. coli* respectively. Inhibition of zone formation was due to the release of ciprofloxacin from the micro-domain structured porous scaffolds and thus facilitated the spontaneous diffusion of ciprofloxacin in nutrient medium.

5. CONCLUSIONS

Ciprofloxacin microspheres impregnated 3-D scaffolds were fabricated successfully employing emulsification freeze drying technique. The collagen scaffold exhibited its supremacy in all respects of drug delivery systems compared to other scaffolds. Majorly, the collagen

scaffold exhibited drug release was immediate with an initial burst release that can be expected to facilitate in wound healing process. The collagen scaffold possessed synergized antibacterial activity due to presence of ciprofloxacin. The outcome of study indicated that wound healing treatment with an antibacterial agent loaded collagen based biomaterials are certainly advantageous over conventional methods. Overall, the outcome of this work introspects that collagen based biomaterials can contribute significantly in tissue engineering and regenerative medicine, since their ultimate biocompatibility and ultra low immunogenicity.

Conflicts of interest

The authors declare that this article content has no conflicts of interest.

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