

Accepted Manuscript

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PII: S0928-4931(16)32221-4

DOI: doi: [10.1016/j.msec.2017.03.110](https://doi.org/10.1016/j.msec.2017.03.110)

Reference: MSC 7621

To appear in: *Materials Science & Engineering C*

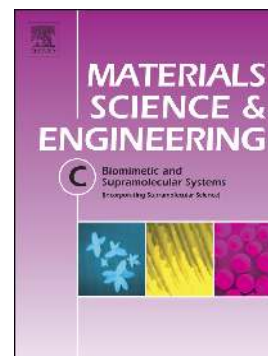
Received date: 18 November 2016

Revised date: 2 January 2017

Accepted date: 13 March 2017

Please cite this article as: Anindita Laha, Chandra S. Sharma, Saptarshi Majumdar , Sustained drug release from multi-layered sequentially crosslinked electrospun gelatin nanofiber mesh. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. *Msc*(2017), doi: [10.1016/j.msec.2017.03.110](https://doi.org/10.1016/j.msec.2017.03.110)

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**Sustained Drug Release from Multi-layered Sequentially Crosslinked Electrospun
Gelatin Nanofiber Mesh**

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ABSTRACT

The aim of this study is to develop electrospun gelatin nanofibers based drug delivery carrier to achieve controlled and sustainable release of hydrophobic drug (piperine) for prolonged time. To accomplish this, we devised some strategies such as sandwiching the drug loaded gelatin nanofiber mesh with another gelatin nanofiber matrix without drug (acting as diffusion barrier), sequential crosslinking and finally, a combination of both. As fabricated multilayered electrospun nanofibers mesh was first characterized in terms of degradation study, morphology, drug-polymer interactions, thermal stability followed by studying their release kinetics in different physiological pH as per the gastrointestinal tract. Our results show that with optimized diffusional barrier support and sequential crosslinking together, a zero order sustained drug release up to 48 h may be achieved with a flexibility to vary the drug loading as per the therapeutic requirements. This work lays out the possibility of systematic design of multilayer nano-fiber mesh of a biopolymer as a drug delivery vehicle for hydrophobic drugs with a desired signature of zero order release for prolonged duration.

Keywords: Gelatin nanofiber; Hydrophobic drug; Zero order release; Crosslinking; Multi-layers

1. Introduction

Over the years, researchers have engineered different drug-delivery vehicles to maintain the drug concentration in the body within its therapeutic range for prolonged time [1-8]. Electrospun nanofiber is also one of such systems that have dragged lot of attentions in recent past. Exceptional properties of such fibers, e.g. highly porous three dimensional surface, high surface-to-volume ratios, interconnected porosity with tuneable pore dimensions, found tremendous applications in different biomedical fields [9-15]. Various electrospinning parameters modulate the fiber diameter and thickness, which may affect sustained and controlled release profiles [16-18]. To increase the diffusional path between the drug and dissolution medium, eventually the concept of multi-layered electrospun fibers was developed, which enabled better control in release [13, 14]. The idea behind preparing multi-layered electrospun mesh is to get sustained molecular release for a prolonged time by controlling the drug mobility. By sandwiching the drug loaded layer between two adjacent electrospun layers, one can control the kinetics of water uptake, which in turn promotes the sustained release of drug molecules through precise control in both degradation as well as osmotic pressure [13, 14]. However to the best of our knowledge, there is no such study being performed for hydrophobic drugs, delivery of which is a challenge for any biodegradable polymer.

In our previous work, we fabricated single layered piperine loaded (model hydrophobic drug) electrospun gelatin nanofibers (GNF) mesh, and achieved sustained release to some extent as per the gastrointestinal (GI) tract, by controlling the swelling and crosslinking [19]. Due to the highly porous structure of nanofibers, we have crosslinked GNF using saturated vapor of glutaraldehyde (GTA) because of its tremendous capability to crosslink

within a short time span [19]. We were able to crosslink the mesh by only a few minutes of GTA exposure (6 min) instead of a few hours or even days as reported in literature [20-22]. This might reduce the risk of toxicity caused by GTA. Results showed a typical two stage, curved profile for all the cases i.e. a sudden release of drugs during initial hours followed by a much slower release during the rest of the observation time scale. The probable reason for this kind of common release pattern could be the penetration of water molecules in the mesh which caused swelling of the matrix. Due to swelling, the drug molecules from the outer layers started releasing rapidly, which caused sudden release (65 %) of drug molecules from 6 min crosslinked mesh (G-P NF C6) within 4 h in pH 7.4 [19]. This kind of profile can be used for specific treatment purpose where initial release of drug is required. However in general, this type of release profile potentially can cross the toxic drug-concentration level within initial hours and sub therapeutic drug concentration in later part [23]. As piperine is the most formidable bio-enhancer till date, it helps to enhance the bio-availability of other drugs by boosting the absorption from the intestine [24]. It also has anti-microbial, anti-inflammatory, anti-depressant, anti-cancerous effects [25-27] apart from being hydrophobic in nature. This motivated us to further modify our electrospun GNF vehicle by introducing multi-layered sandwiched mesh structure, so that it can achieve close to zero order/controlled release in intestine. Here, piperine was incorporated into sandwiched GNF mesh with variations in barrier and core layer thicknesses. The effect of drug concentration and sequential crosslinking on release profile was also investigated. In addition to that, controlling the mobility of hydrophobic drug molecules through hydrophilic polymer matrix is also challenging [19]. Therefore, the objective of this work is to regulate the release profiles in such way so that

we can achieve almost zero order release (without initial burst release) of piperine (a model hydrophobic drug) for nearly 48 h from this newly developed vehicle.

2. Materials and methods

2.1. Materials

Gelatin (Type A, 175 bloom), piperine (98%), hydrochloric acid (ACS, 36.5-38.0%), glutaraldehyde (25% v/v aqueous solution), acetic acid (glacial, ACS, ~99.7%), sodium hydroxide pellets (98%), phosphate buffer saline (pH 7.4) were purchased from Alfa Aesar. Deionized water (DI) (Milli Q, resistivity 18.1 M Ω .cm) was used throughout the experiments.

2.2. Fabrication of nanofiber membrane

Multi-layered meshes were prepared by electrospinning 20% (w/v) of Gelatin (Type A) solution in acetic acid (20% v/v in distilled water) solvent using electrospinning apparatus (Make: E Spin Nanotech Pvt. Ltd, India). Then a known amount of piperine was added to spinning solution for drug loaded samples (G-P NF). Multi-layered GNF was prepared by sequential eletrospinning of with and without drug loaded solutions on the substrate (aluminium foil). The samples were then crosslinked using saturated vapor of GTA (25% v/v aqueous solution) for few minutes (6 and 8 min; i.e. G-P NF C6 and G-P NF C8 respectively) [19].

2.3. Characterization

The morphology of the fibers was examined by table top Scanning Electron Microscopy (SEM) (Make: Phenom world ProX, Netherlands). To reduce the charging effect, samples were coated with thin gold layer using sputter coater (DC Sputtering system, Make: Excel

Instruments, India). *In-vitro* degradation studies were then carried out to check the stability of the crosslinked membranes (G-P NF C6 and G-P NF C8) in PBS (pH 7.4) at 37 °C for 48 h. The weight loss (WL %) due to hydrolytic degradation is calculated by using the following equation [9]: $\text{Weight loss (WL \%)} = \left(1 - \frac{M_f}{M_i}\right) \times 100$ Where, M_f = Sample mass after an incubation period. M_i = Initial sample mass. Similarly, Swelling degrees (SD) were calculated using the following equation [9]: $\text{Swelling degree (SD \%)} = \frac{(W_s - W_d)}{W_d} \times 100$; where, W_s = Weight of swelled sample and W_d = Initial weight of dried sample. To investigate the stability of the drug and the effect of cross-linking on the mesh, samples were characterized by Fourier transform infra-red spectroscopy (FTIR, Bruker Tensor 37, USA) in 400-4000 cm^{-1} range with a resolution of 4 cm^{-1} and 256 scans per samples. Thermal stability of the vehicle was investigated by thermogravimetric analysis (TGA) (Model: Pyris 1, PerkinElmer Inc., USA) in helium atmosphere in the range to 35 to 600 °C at a heating rate of 10 °C/min. Finally, *in-vitro* release of the drug i.e., piperine from multi-layered G-P NF, meshes were analysed using UV-vis spectroscopy (Lambda 35 Perkin Elmer, USA) at 342 nm i.e. the λ_{max} for piperine [19]. To investigate the drug release kinetics and mechanism, the *in-vitro* release data were analysed using zero order equation [23].

3. Results and discussion

3.1. Surface morphology analysis

The primary challenge of most of the drug-delivery systems is to achieve nearly zero order release profile for prolonged duration. To meet such requirements using GNF,

multi-layered mesh was fabricated and finally examined in terms of morphology, water resistivity, degradation, chemical and thermal stability as well as release studies.

The surface morphology of electrospun G-P NF, G-P NF C6, G-P NF C8 samples are represented in Fig.1. Randomly oriented continuous piperine loaded gelatin nanofibers are presented in Fig.1a. Nanofiber mesh is highly porous structure and gelatin is highly soluble in water. Due to such high ratio of surface area to volume, this kind of porous structure with large surface area will immediately dissolve in contact of water molecules in any types of aqueous solutions. Thus for crosslinking the gelatin fiber, we used saturated vapor of the same solution (25% v/v aqueous solution of GTA) to minimize the water related degradation of gelatin fibers. Here, the membranes are thus crosslinked with saturated vapor of GTA for 6 and 8 min, presented in Fig.1b and 1c. Water molecules present in saturated vapor have partially degraded the fibers and fused them together shown in Fig.1b and 1c (more compact morphology). The partial degradation due to swelling of fibers is commonly visible in case of gelatin nanofibers which are reported in our previous work [19]. Due to the presence of hydrophobic molecules (piperine in this case) in the gelatin mesh, the fusions of fibres are substantially less. This fused structure of nanofiber membrane can tailor the release of drug molecules to the release medium.

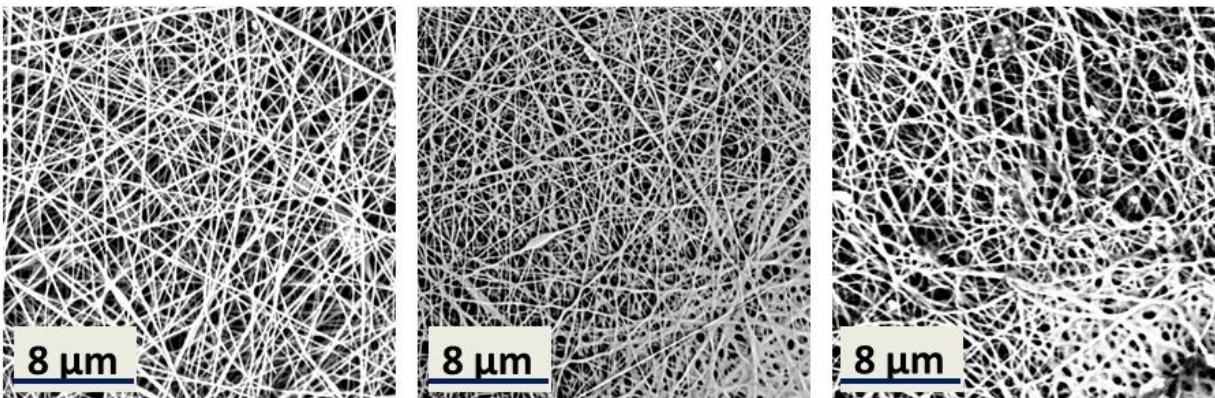


Fig. 1. Morphology of the electrospun fiber membrane of a) G-P NF, b) G-P NF C6 and c) G-P NF C8

3.2. *In-vitro* degradation study analysis

To understand the effect of crosslinking on G-P NF samples *in-vitro* degradation study and swelling study were performed in PBS (pH 7.4: mimicking physiological pH of intestine) only for 50 h (Fig. 1S). Although, the crosslinked mesh need to sustain a period of maximum 4 h in pH 1.2 (mimicking physiological pH of stomach), we checked the stability for 24 h in our previous work [19]. Results showed that G-P NF C6 was reasonably stable in aqueous medium for a period of 50 h and therefore considered for further studies. In summary, we have successfully crosslinked the mesh with minimal exposure in GTA vapor which can sustain more than 2 days in physiological pHs.

3.3. Thermal and chemical stability analysis

TGA thermograms of samples showed two stages of weight loss inside the overall range of 35-700 °C and are presented in supplementary section (Fig. 2S). Weight loss for G-P NF C6 was found to be less in compare to G-P NF, which is an indication of improved thermal stability upon crosslinking.

After checking thermal stability, chemical stability of drug loaded gelatin nanofiber fabricated vehicle was also investigated. To understand the crosslinking effects on nanofibers and the co-existence of both drug and polymer with their own characteristic identify, FTIR was performed and results were presented in supplementary section as Fig. 4S [19]. The shifting of peaks near amide I, II and III as an after-effect of 6 min crosslinking were also reported [19]. Comparison between pure gelatin fiber and piperine loaded gelatin fibers were detailed in previous work [19]. In a nut shell, the presence of amide peaks of gelatin around 1628 cm^{-1} and aliphatic C-H stretching of piperine around 2920.71 cm^{-1} proves chemical stability of piperine in gelatin nanofiber mesh.

3.4. *In-vitro* release study analysis

After investigating the different aspects of the vehicles such as: the drug-polymer interactions, thermal stability, swelling and degradation in aqueous medium, *in-vitro* drug release was done in order to design a nanofiber based vehicle which can provide a constant drug release for a prolong period of time.

3.4.1. *Designing the carrier:*

The intention of this study was to develop a drug delivery vehicle, which can provide a near to zero order release profile in different physiological pH. Thus, we started modifying our existing single layered vehicle which can successfully circumvent the two stage release profile.

(a) *Effect of multi-layer in release profile*

First, we attempted to design a vehicle with sufficient diffusional barrier which can exhibit a close to zero order release without initial fast release of drug molecules. To meet the aforementioned release profile for the cases with appreciable drug loading, we fabricated

different types of sandwiched structured multi-layered mesh by coating drug loaded layers by two sequential layers of gelatin as shown in Fig. 2 and Table 1.

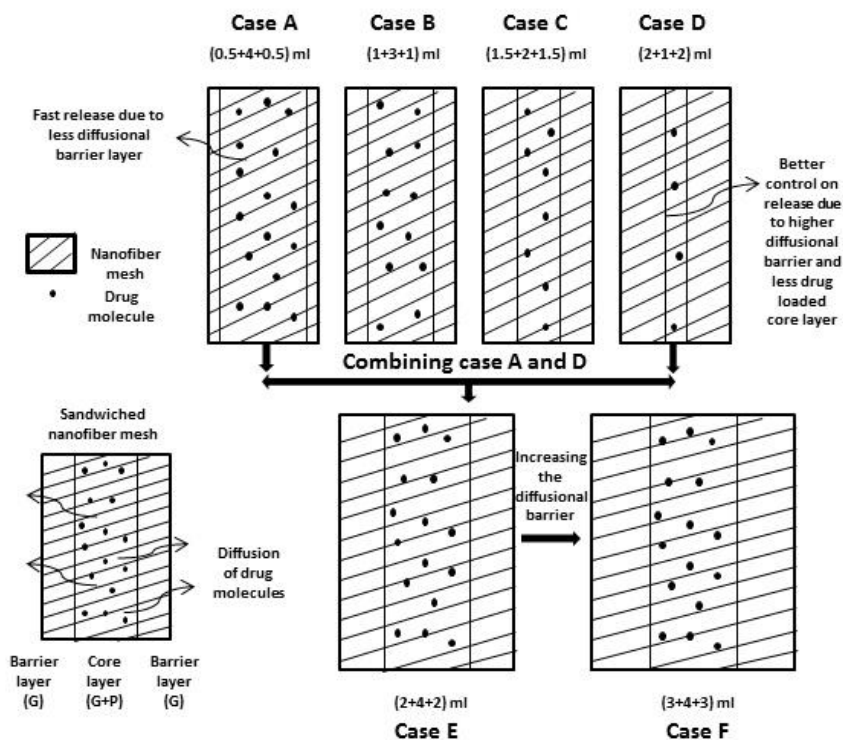


Fig. 2: Schematic presentation different composition of sandwiched nanofiber mesh.

Table 1: Summary of the configuration of sandwiched electrospun nanofiber membrane

Cases	Composition G-(G+P)-G (layers) (ml)	Remarks based on the drug release profiles (Fig. 3a)
A: G-P NF C6/4/0.5	(0.5+4+0.5)=5ml	1. G Barrier is very less (0.5 ml). 2. G+P is sufficient (4 ml)
B: G-P NF C6/3/1	(1+3+1)=5ml	1. G Barrier better than A.
C: G-P NF C6/2/1.5	(1.5+2+1.5)=5ml	1. G Barrier is improved (1.5 ml) 2. G+P is less (2 ml)
D: G-P NF C6/1/2	(2+1+2)=5ml	1. Good diffusional barrier (2 ml) 2. Very less drug loading (1 ml)
E: G-P NF C6/4/2	(2+4+2)=8ml	1. Combining A and D 2. Good amount of drug loading (4ml) 3. Sufficient diffusional barrier (2ml)
F: G-P NF C6/4/3	(3+4+3)=10ml	1. Further improved sample E by adding extra diffusional barrier (3ml) 2. Drug loading is same (4ml)

As a starting point, 5 ml of polymer solution was deposited to fabricate four different sandwiched structure i.e. A to D (Table 1 and Fig. 2). Fig. 3a showed initial fast release of drug (within 4 h) for first four cases (A to D) decreased (A: $52.0 \pm 5.5\%$ to D: $15.6 \pm 3.7\%$ within 4 h) drastically with an increase of diffusional barrier from 0.5 ml to 2 ml in both sides of the core

layer. On the other hand the core layer has gradually decreased (4 ml to 1 ml) affecting overall release profile particularly for case D. Therefore, we have combined both A and D formulations to design a new formulation E (total solution 8 ml) shown in Table 1 and Fig. 2. Thus, the new formation E consists of 4 ml core layer similar to sample A and 2 ml barrier layers from both sides similar to sample D. More control on the mobility of drug molecules was noticed with this newly fabricated sample E. From Fig. 3a, it can be observed that case E has much control in initial release during first 4 h with an appreciable overall release in 24 h. Further to fine tune the initial fast release of the drug molecules, additional barrier layers were added to sample E, which has resulted in the fabrication of sample F shown in Table 1 and Fig. 2. In that case, a total 10 ml of polymer solution was deposited to fabricate the membrane with enough drug loaded core (4 ml) and good diffusional barrier (3+3 ml) to control the release profile (Sample formulation: F). Release profile (Fig. 3a) for sample F exhibited a good control over the mobility of drug molecules and showed a sustained drug release ($22.5 \pm 6.5\%$ in initial 4 h; total $55.9 \pm 2.9\%$ in 24 h). Thus further investigation was done with sample F to check the effect of pH (pH 1.2, 6.8 and 7.4; similar pH profile of human GI tract) and the effect of drug concentration (1.5, 2, 2.5, and 3.5 mg/ml) on release profiles.

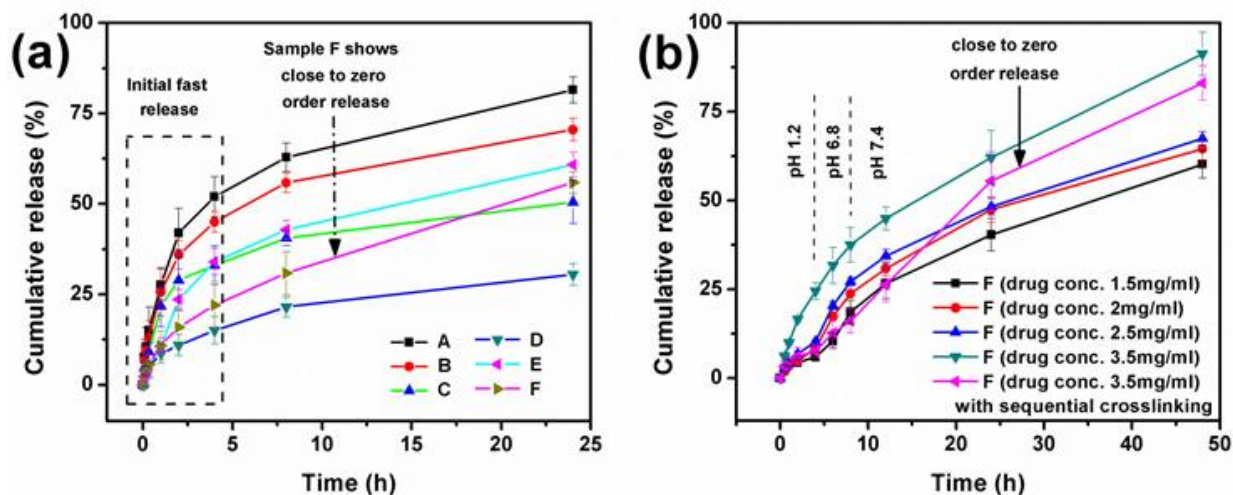


Fig. 3: a) *In-vitro* cumulative release of piperine from different composition of sandwiched membranes. b) Cumulative release of piperine from F sample with different drug concentration (1.5, 2, 2.5, 3.5 mg/ml) at pH 1.2 for 4 h, then pH 6.8 for 4 h and finally pH 7.4 for 16 h. (results represented are mean \pm SD , n=3)

(b) *Effect of pH and drug concentration on release profile*

To modify our selected vehicle F based on the results discussed above, the *in-vitro* system was designed in such a way so that the vehicle could be exposed to different pH with different retention time, as similar to GI tract [23]. The intension of the design such *in-vitro* system was to understand the effect of swelling and degradation of the vehicle during drug release. Effect of pH on the release of piperine from sample F with different drug concentration (1.5, 2.0, 2.5 and 3.5 mg/ml) was showed in Fig. 3b. The release of drug molecules is accelerated with increase in pH for all the cases. This accelerated release can be explained by the increase in swelling degree in higher pH. In higher pH, all the $-\text{COOH}$ groups present in gelatin converts into $-\text{COO}^-$ which results high anion-anion repulsion and thus high swelling of the polymer matrix. But in acidic pH (<5), due to the high ionic strength of medium, most of the carboxylate groups are protonated

and the anion-anion repulsion force are minimized [28]. Additionally, the hydrogen bonding between carboxylate and hydroxyl group is also strengthened, which causes overall shrinkage and lesser swelling in acidic pH. Thus, swelling capability increases gradually with increase in pH which promotes better drug release in higher pH conditions [23]. This phenomena can help to minimize the drug loss in lower pH of GI tract (pH of stomach is 1.2 and retention time is approx. 4 h) and can effectively swell as well as deliver maximum drug in higher pH region (pH of different parts of intestine are 6.8 and 7.4 respectively). Fig. 3b reveals that the drug loading in core layers is proportional with drug release. Thus increase in initial drug release within 4 h is observed with higher drug concentration in core layers: $6.0 \pm 1.2\%$, $8.4 \pm 1.1\%$, $10.3 \pm 0.5\%$, and $24.5 \pm 2.3\%$ of cumulative release for 1.5, 2.0, 2.5 and 3.5 mg/ml respectively. The F sample with 3.5 mg/ml drug concentration didn't seem to be promising to reach zero order drug release due to initial fast release compare to other samples. Although, the highest loaded sample showed initial rapid release of drug within 4 h, we have selected the vehicle for further modification in order to overcome the drawback associated with burst release while still maintaining the higher drug concentration. Our objective is to simultaneously reduce drug loss in stomach (pH 1.2) within initial hours (approx. 4 h) and to release drug molecules in the intestine (absorption site) in a zero order manner with F sample with highest possible drug loading, i.e. 3.5 mg/ml. In order to achieve that, we then worked on another strategy, sequential crosslinking, as discussed further.

(C) Effect of sequential crosslinking on release profile

We now investigated the effect of different crosslinking methods on the chosen vehicle (3.5 mg/ml and F type) in order to overcome the limitations of F sample (sudden release in initial hours with higher drug loading). Thus, sequential crosslinking of the vehicle was attempted as an

additional step. To understand this further, we first deposited the barrier layer of gelatin (3.0 ml) nanofibers and then crosslinked it for 2 min with GTA vapor followed by deposition of drug loaded core layer (4.0 ml) and again crosslinking for 2 min followed by deposition of only GNF (3.0 ml) and then again crosslinking for 2 min. The crosslinking was done sequentially keeping the total crosslinking time as 6 min. A comparative study of release profile between sequential crosslinked and one time crosslinked sample, both with 3.5 mg/ml F sample, was made in Fig. 3b. The release of drug from sequential crosslinked sample with 3.5 mg/ml of drug after 4 h was less than 10% ($7.6 \pm 1.8\%$), whereas one time crosslinking with same drug concentration, the initial release within 4h was almost 25% ($24.5 \pm 2.3\%$). Results showed sequentially crosslinked sample with 3.5 mg/ml of drug successfully toned down the drug loss during initial hours in lower pH conditions. At the same time, it showed controlled and sustained release of piperine ($83.0 \pm 4.8\%$ of release after 48 h) for rest of the observation time scale.

The probable reason of these observations can be understood by the uniformity of crosslinking of fibers in-between layers of the mesh while doing it in sequential manner. The compactness of the vehicle has increased due to the layer-by-layer crosslinking strategy which also elevated the water resistivity degree and restricted the drug molecule mobility [13-15]. Further to understand the mechanism of drug release, *in-vitro* drug release data for sample F with different drug loading and different crosslinking strategies were fitted with zero order release and R^2 values were listed. Final design was giving R^2 as 0.99 for 24 h and 0.97 for 48 h release, which is a signature of a zero order case for prolonged time. This has been presented in supplementary section (Table 1S and Fig. 5S). Thus it is worthwhile to mention that by doing a systematic analysis, the final vehicle design has achieved an optimal performance i.e. much control in initial release profile without compromising on overall drug release along with important aspects like

stability and less release in lower pH as well as desired zero order release profile in higher pH condition for 48 hours using a very cheap biopolymer gelatin based nanofibers.

4. Conclusion:

Biodegradable polymer mesh was fabricated by electrospinning of natural polymer gelatin solution with different concentration of drugs for the assessment of a polymeric drug delivery system with hydrophobic drug molecules. In order to get close to zero order drug release, multi-layered membranes with different drug concentrations and different crosslinking strategies were applied. The effect of crosslinker was investigated in terms of degradation, swelling, chemical stability and thermal stability. Finally, in-vitro release study of the vehicle was done in different physiological conditions mimicking the pH profile of GI tract. In order to control the initial fast release, different combinations of multi-layered membranes were fabricated and studied. As a next step, by modifying the core as well as the barrier layer and the crosslinking methods, we have demonstrated that one can fabricate electrospun nanofiber mesh which can exhibit better control over the initial fast release of a hydrophobic drug in a substantial level and can achieve close to zero order release profile for 48 h with flexibility to vary drug loading as per the therapeutic requirements. This work lays out the possibility of systematic design of multilayer nano-fiber mesh of a cheap biopolymer (gelatin) to be used as a drug delivery vehicle for hydrophobic drugs with a desired signature of zero order release for long hours.

Acknowledgements

The authors acknowledge to Indian Institute of Technology Hyderabad for providing necessary research infrastructure to carry out this work. Authors also acknowledge DST Nano-Mission (Dept. of Science and Technology, Nano-Mission Committee, Govt. of India) for necessary funding to this research area.

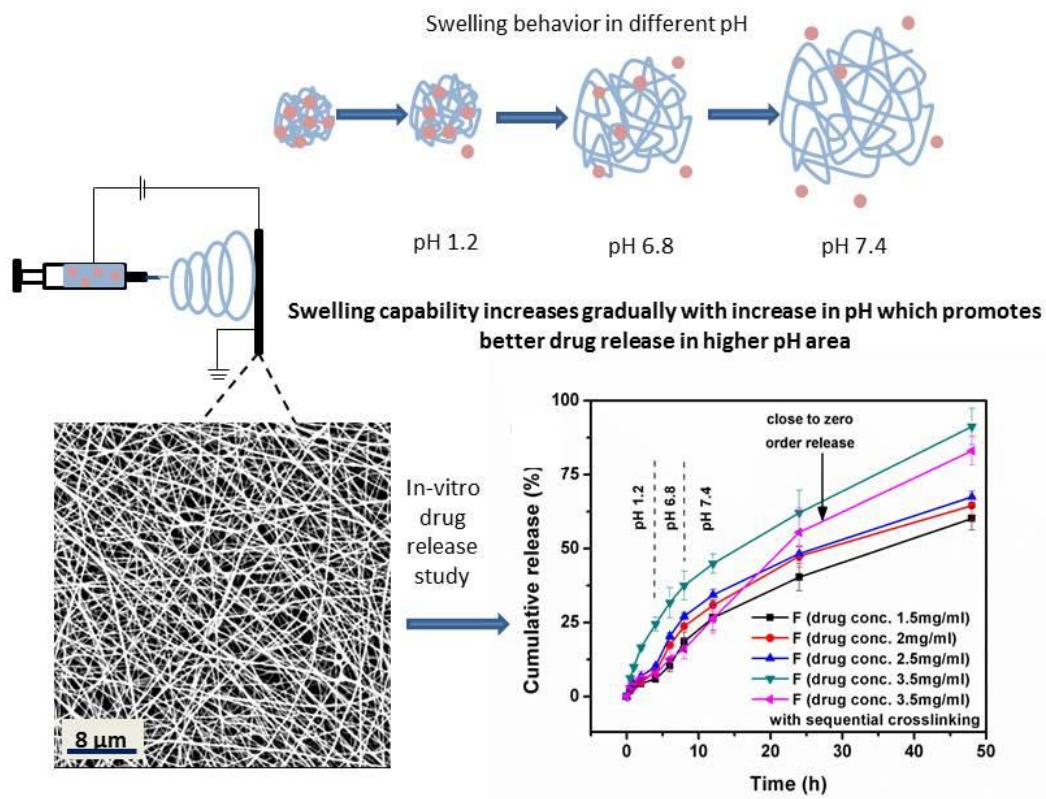
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Graphical abstract

Highlights:

1. Multi-layered electrospun gelatin nanofiber mat was used as a vehicle for drug delivery
2. Effect of barrier and drug loaded core layer is directly related to release profile.
3. Sequential crosslinking achieved better uniformity resulting controlling initial burst release
4. Zero release profile of piperine observed up to 48 h at different physiological pH.

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