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DESIGN AND DEVELOPMENT OF A PISCINE COLLAGEN BLENDED PULLULAN HYDROGEL FOR SKIN TISSUE ENGINEERING

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This present study was designed to prepare a superabsorbent tailor made collagen-pullulan hydrogel with improved mechanical stability and well-defined biocompatibility for skin tissue engineering. Three-dimensional scaffolds (3D) was fabricated by using natural polysaccharide pullulan, cross-linked with sodiumtrimetaphosphate (STMP) and blended with collagen to form a polymeric network. Collagen was extracted from the skin of unexplored puffer fish *(Lagocephalus inermis*). The cross-linking occurred at alkaline pH at room temperature to give translucent, clear and soft hydrogels. Swelling studies revealed remarkable water absorption property with swelling ratio up to 320%, an ideal characteristic for the hydrogel to provide a moist wound healing environment. SEM analysis revealed highly interconnected porous structure of the collagen blended pullulanhydrogels. MTT assay performed on NIH3T3 fibroblast cell lines revealed that the prepared hydrogels were 100% biocompatible with enhanced cell adhesion and proliferation. Hydrogels promote angiogenesis in the chick chorioallantoic membrane was investigated by the CAM assay. Wound healing studies exhibit statistically significant (< 0.05) response to wound contraction and re-epithelialization in excision wound model in rats. Compared with 49% wound closure in 11±2 days in controls, 96% wound closure was observed in rats treated with collagen-pullulan hydrogel (CGPNH). The highly porous collagen-pullulan hydrogels were successfully developed with a significant in vitro and in vivo biological performance, a promising biomaterial for wound healing applications

Introduction

Hydrogels are superabsorbent three- dimensional (3D) macromolecular networks of hydrophilic polymers which play significant roles in biomedical applications, especially in skin repairs and tissue engineering. The 3D integrity of hydrogels in their swollen state is maintained by either physical or chemical cross-linking[1]. The remarkable swelling property of hydrogel is mainly due to its network elasticity and porosity. Hydrogels can absorb and release water in a reversible manner, in response to specific environmental stimuli viz.,temperature, pH, ionic strength etc.[2] It is possible to change the chemistry of hydrogel by controlling their polarity, swelling behaviours, surface, and mechanical properties. Such a smart behaviour towards the adjustment of physiological variations promotes them as a potential candidate for wound healing applications.[3]Natural biodegradable polymers are generally hydrophilic in nature with excellent biocompatibility and weak immunogenicity.[4] Since the studies on natural polymers are always fascinating and interesting area of research for the development of newer products, and for this purpose polysaccharides and proteins have been extensively investigated for dermal reconstructions. Theblending of natural polymers will produce a potentially active biomaterial with tailored properties without destroying their original properties.

Pullulan, an excellent polysaccharide produced from the yeast like fungi *Aureobasidiumpullulans*is composed of linear α-(1→6) linked maltotriose units and a small number of α-(1→4) linked maltotetraose units. It is biodegradable in biologically active environments, it forms easily thermo-stable, transparent, flexible films with an extremely low oxygen permeability.[5] It has good adhesive and binding properties. As a hydrophilic polymer, it has the unique characteristic of excellent swelling properties, which make them as a suitable candidate for various biomedical applications, especially in dermal tissue engineering[6].

Collagen, an interesting polymer with unique structure, size and amino acid sequence having wide range applications in biomedical, pharmaand cosmetic industries. It is a major component of extracellular matrix (ECM) and one of the most abundant proteins in mammalian tissues. Collagen can also form fibres with extra strength and stability, through its cell aggregation and cross-linking

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property[7]. However, its applicability is limited owing to its high cost and the probability of disease transmission from the bovine sources[8]. Recent studies proved that the collagen derived from marine sources were much safer and did not produce any immunological response or allergic reactions when compared to mammalian tissue collagen[9].Thus, in the present study, collagen extracted from the skin of puffer fish *Lagocephalusinermis,*(a novel marine source) was studied for the preparation of hydrogel based system. This is the first paper reporting the extraction of collagen from this underutilized fish from Indian origin belongs to the family Tetraodontidae. In this study, we aimed to develop an effective porous biomimetic hydrogel from fish skin collagen blended with pullulan for wound healing applications.

Materials and Methods

Collection of Fish skin

The smooth golden puffer fish *Lagocephalusinermis* was collected in the month of July 2014, from Mallipatinum seashore, Tamil Nadu, India and were brought to the laboratory at 4 °C. The skin was removed from the fish and immediately washed with distilled water to remove the blood stains and debris and cut into small pieces approximately 5 x 5 cm and stored at -20 °C until further used.

Chemicals

Pullulan (molecular weight 200,000), sodiumtrimetaphosphate (STMP), Collagenase A (from *Clostridium histolyticum*) and pullulanase were purchased from Sigma-Aldrich, India. Until and unless mentioned all the chemicals and reagents used in this experiment were obtained from Sigma-Aldrich, India. Deionized water was used throughout the studies. NIH3T3 fibroblast cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India.

Extraction of collagen from fish skin

Acid soluble collagen was extracted from the puffer fish skin as per the method of Nagai and Suzuki (2000)[10] with slight modification.To remove non-collagenous proteins, the fish skin was mixed with 0.1 M NaOH at a ratio of 1:10 (w/v).The mixture was then continuously stirred for 6 h at 4 °C and thenwashed with cold water until neutral pH obtained. Pre-treated skins were defatted with 10% butyl alcohol for 48 h and the solvent was changed every 6 h. The defatted skin was treated with 0.5 M acetic acid for 24 h. The mixture was filtered through two layers of cheese cloth and supernatant was separated out. Salting out of the collagen from the supernatant was achieved by using of 0.9M NaCl and it was kept undisturbed for 24 h at 4°C. Then the suspension was centrifuged at 8000 rpm for 1 h at 4 °C. Then the precipitate was dialyzed against 0.1M acetic acid and distilled water for 24 h each, respectively. The final extracted product was acid soluble collagen.

Hydrogel fabrication

Hydrogel fabrication was done according to the method of Dulong, with slight modifications[11]. For this study, five hydrogel formulations were prepared, all of which contained a constant pullulan concentration (2g). The polymer and cross-linking concentrations of different blends are shown in table 1. For the synthesis of Pullulan hydrogels (PNG, PNS, and PNH), 2g of pullulan was mixed with different concentrations (1, 1.5, 2g, respectively) of STMP in 10ml of deionized water. Finally, 50µl of 0.1M NaoH was added to the mixture and stirred well for 90 min at room temperature until the transparent viscous liquid was produced. This transparent viscous liquid, then underwent a sol-gel transition, when incubated at 50°C for 30 min.

For the synthesis of collagen-pullulan hydrogels (CGPNH, CGPNH1), different concentration of collagen (0, 0.1 and 0.2g) was mixed with 2g of pullulan and 2g of STMP in 10ml of deionized water with constant stirring, respectively. The pH was adjusted to 9.0 using 0.1M NaOH and the composite mixture was stirred well for 90 min at room temperature to promote the homogenous distribution of polymers within the hydrogel. The mixture was poured into the petridishes and incubated at 50°C for 30min for the formation of hydrogels. A circular punch was used to cut 6-mm diameter gels. The prepared hydrogels were washed extensively with phosphate buffer saline until neutral pH obtained and stored at 4°C until further use. Hydrogels were sterilized overnight under UV light prior to all experiments.

Table.1 Pullulan and Collagen–pullulan hydrogel compositions

Hydrogels	Sample	PN (g)	STMP (g)	CG (g)	NaOH (µl)
Pullulan hydrogels	**PNG**	2	1	0	50
	PNS	2	1.5	0	50
	PNH	2	2	0	50
Collagen – pullulan hydrogels	CGPNH	2	2	0.1	50
	CGPNH1	2	2	0.2	50

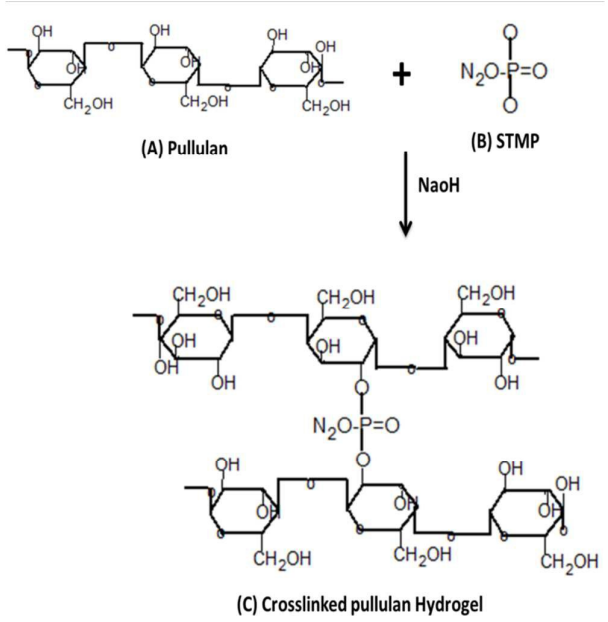

Fig.1 Pullulan cross-linked with STMP under alkaline condition

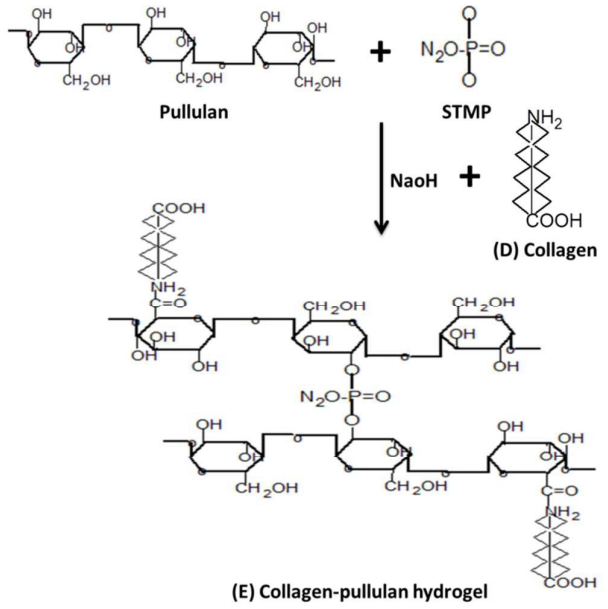

Fig. 2. Synthesis of collagen- pullulanhydrogel (CGPNH)

Evaluation of formulated hydrogels

Appearance

The hydrogels formulated were observed for their visual appearance, color, the texture feels upon applications such as smoothness, stiffness, and its stickiness.

pH

The pH of the hydrogelshas also played an important role in the formation of a gel. The pH of the hydrogels was determined by the pH meter. The determinations were carried out in triplicate and the average of three readings was recorded.

Viscosity

The viscosities of formulated hydrogels were determined using Brook- field viscometer (spindle number LV-16) in triplicate and the average of three readingswas recorded.

Physico-chemical characterization of hydrogels

Fourier transforms infrared spectroscopy (FTIR)

FTIR analysis was done in the range of 500– 4000 cm^{-1} with resolution 4 and 128 times scanning using Nicolet Magna-IR 560, USA.

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed using high resolution 2950 TGA thermo gravimetric analyzer (TA instruments Co., Austria). Samples weighing between 10 and 20mg were placed in a platinum pan and test was carried out in a programmed temperature range of 0 - 800˚C at a heating rate of 5˚C/min under a nitrogen atmosphere at a flow rate of 50 ml/min.

Differential Scanning Calorimetry (DSC)

The thermal characteristics of hydrogels were investigated using a differential scanning calorimeter (TA instruments Co., Austria) in order to measure their crystallization temperature (TC) and melting temperature (Tm). The analysis was performed at a heating rate of 20°C/min, from 30°C to 200°C, under nitrogen gas at a flow rate of 10 ml/min.

Swelling Studies

The degree of swelling could be characterized as water retention ability of the hydrogel. Small pieces ($2cm^2$) of PNHand CGPNH hydrogels were weighed and soaked in phosphate buffered saline (pH 7.4) at 37°C. Excess liquid was gently shaken off and weights of

swollen gels were obtained. Hydrogel weights were measured at 6, 12, 18, 24, 48, 72 h. Six samples were tested for each condition. Then, the swollen products were dried at 37 °C under vacuum to a constant weight. The equilibrium percentage of swelling (% swelling) of the hydrogels was calculated as per Eq. 2

$$\% \text{ of Swelling} = [(W_s - W_d)/W_d] \times 100$$

Where W_s is the weight of the product after hydration for 72h and $W_d$is the weight of the dried product.[13,14]

In vitro biodegradation studies

The in vitro biodegradation of PNH and CGPNH hydrogels were carried out according to previously described procedures[15–17]. The PNH and CGPNH hydrogels were incubated with collagenase A(2mg/ml), pullulanase (4U/ml) in PBS and weighed every 12h for 7 days. Combination degradation studies using both the enzymes were conducted in PBS using the same concentration as above. The initial weight at time 0 was the dry weight of the hydrogels (W0), and after being degraded by the enzymes, the wet hydrogels were washed three times with water (W1). These hydrogels were used for subsequent measurements. The experiments were performed in triplicates under each condition at 37^{o} C. The weight loss (%) was calculated as follows(Eq.3);

$$\% \text{ of weight loss} = (W_0 - W_1) / W_0 \times 100$$

Morphology observation

The internal structure of the hydrogels was investigated by Scanning electron microscopy (JSM-5600LV, JEOL). The hydrogels were dehydrated by a freeze dryer and sputter coated with goldbefore SEM characterization

Heamocompatibility test

The hemostatic potential of the hydrogels was determined using[17]human blood according to Pal et al., with slight modifications.Percentagehemolysis was calculated as per the following formula (3)

$$\% \text{ Hemolysis} = \{(OD_{test} - OD_{negative}) / (OD_{positive} - OD_{negative})\} \times 100$$

Where, ODis the optical density.

Biocompatibility

Biocompatibility was assessed using MTT [3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] assay. The samples were placed on polystyrene petriplates and were sterilized with ethanol-UV treatment. This was followed by seeding with 20µl of NIH3T3 fibroblast cell suspension (4X105 cells/ml) on the samples and incubated at 37°C for 1 h to allow cell adherence to the surface of the samples. Thereafter, the samples were incubated for another 2 days. The cell viability was measured after 24 h and 48 h using MTT assay. MTT was added at the end of the incubation period and the plates were incubated for 4 h at 37°C. Following incubation, the media was aspirated completely and MTT formazan crystals formed were dissolved by the addition of dimethylsulfoxide (DMSO), and the absorbance was read at 570 nm using a multiwell plate reader (Multiskan Ascent VI.24). Untreated wells served as cell control. Data were normalized to control to evaluate the biocompatible potential of hydrogel on cells[18,19].

In vitroChick chorioallantoic membrane assay

The angiogenic property of the hydrogels was assessed by simple and inexpensive CAM assay method. Four-day old chick eggs were obtained and incubated at 37°C and 60% humidity in an egg incubator for 72h. On the eighth day, 2-3 ml of albumen from the egg was aspirated at the acute pole of the egg to create a false air-sac, facilitating its dissociation from the egg shell. A square window of 1cm^2 was cut and lyophilized hydrogel disc was loaded and placed in the junction between two big vessels. The window was resealed and incubated for 72 h, sterilized methyl cellulose disc was used as control[20].

In vivo studies- maintance of animals

All experiments were performed in compliance with the relevant laws and institutional guidelines. Animal study was approved by the Institutional ethical committee (Reg. no: GSP-IAEC- 2013-04- 06) for conducting experiments on animals according to the regulations of CPCSEA, at department of Pharmacology laboratory, GIET School of Pharmacy, Rajahmundry, Andra pradesh, India.

Wound healing potential of the hydrogel was studied by using Wistar albino rats (weighing 150-200g) of either sex. The animals were housed in polyacrylic cages and maintained under standard laboratory conditions (Temp. 25±2°C) with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water ad *libitum*.

Excision wound model

All experiments were carried out upon getting approval from the institutional ethical committee. Rats were inflicted with excision wounds according to the method of Morton and Malone with slight modifications. Animals were divided three groups of six animals in each. Groups-I was served as control. Animals under Group-II and Group III were designed to treat with only PNH and CPNHG hydrogel. An excision wound of 20mm diameter was made on the

pre-shaved, sterile dorsal surfaces of the animal by means of a sterile surgical apparatus under ether anaesthesia in aseptic conditions. The wounds were of full-thickness type extending up to the subcutaneous tissue. The day of wound infection was considered as day zero of the experimental period[21,22].

Healing assessment

The progressive changes in wound area were measured in mm at every 3 days interval. The gradual reduction of the wounded area was measured on 3rd, 6th, 9th, and 12th day after initiation of the wound. Histopathologicalobservations were done using eosin-haematoxylin staining of the tissues and compared to the control tissues for fibroblast proliferation, angiogenesis and collagen synthesis.

Statistical analysis

All the results are given as a mean±standard deviation of three individual experiments (n=3). The comparison between means was done by one-way analysis of variance (P<0.05) followed by Duncan's multiple range analysis using statistical software package SPSS, version 13.0.

Resultsand Discussion

Different hydrogelswere prepared at different cross-linking densities by varying the STMP concentration (Table. 1) at constant NaOH and pullulan concentration (PNG, PNS and PNH). For a low concentration of STMP (PNG & PNS), no cross-linking reaction was occurred properly. The best result was obtained with PNH hydrogel, where the polymer and STMP ratios are equal.Various concentrations (0, 5% and 10%) of extracted fish skin collagen were blended with constant pullulan (2g) and STMP (2g) concentration for the preparation of collagen-pullulan hydrogel (CGPNH, CGPNH1), (Fig.1 and 2). The prepared hydrogels were characterized by its appearance, pH, Viscosity and swelling studies (Table. 2). The prepared PNH and PNH1 gels were non viscous and hard in texture due to the influence of pH and the cross-linking agent (STMP), whereas the CGPNH1 hydrogel showed more viscous and flexible texture. PNH and CGPNH hydrogels were clear, soft, flexible, homogeneous and translucent in nature with extra strength and stability through self-aggregation and cross-linking (Fig.3 and 4). On the basis of the above studies the PNH and CGPNH formulations were found to be more optimum for further in vitro and in vivo studies.

Fig .3. Transparent and soft CGPNH hydrogels

Table : 2 Characterization of formulated hydrogels

Hydrogels	Appearance	Viscosity	pH	Gelation time
PNG	yellowish color, semisolid	3000_{cps}	8.2	60min
PNS	Glossy, yellowish color	3200_{cps}	8.6	45min
PNH	Glossy, clear, transparent gel	4000_{cps}	9.0	20min
CGPNH	Glossy, clear, transparent gel	4600_{cps}	9.0	20min
CGPNH1	Glossy, clear, transparent gel	4800_{cps}	9.0	30min

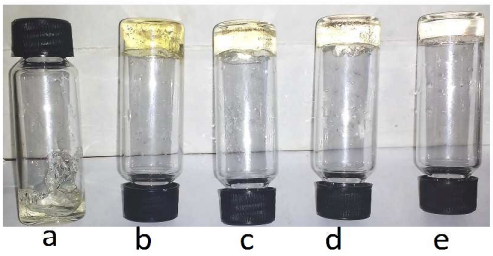

Fig. 4 Different concentration of pullulan and collagen- pullulan hydrogel formation. (a)- PNG- hydrogel not formed (b) PNS (c) - PNH, (D) - CGPNH, and (d) - CGPHN1

Thermal gravimetric analysis (TGA)

Thermal stability of CGPNH hydrogel in comparison with PNH hydrogel and puffer fish skin collagen was characterized using TGA as showed in Fig 5. The thermogram of pure collagen and pullulan hydrogel consists of three degradation stages, while porous CGPNH hydrogel consists of four stages. From the graph, it can be analysed that below 100°C, allthe sample weight loss was mainly due to water loss from the protein and polysaccharide. For collagen and pullulan major weight loss occurred at 60% (in the range 175°-380°C and 255-384°C) and 19% (in the range of 400 - 503°C and 451-580°C) signifies the breakdown or chain cleavage of the organic compounds leads to the rapid weight loss and the formation of gaseous elements[23,17,24,25]. Further, these two peaks assigned to pullulan were observed in the thermogram of the synthesized porous CGPNH hydrogels in the range of 365-490°C of 30% weight loss and 455-520°C of 8%, respectively. These peaks could be assigned to the thermal degradation of polysaccharide rings. At the end of the experiment at 830°C, only 10.11% of the cross-linked CGPNH hydrogel remains as residue.From the above result, it is confirmed that collagen blended with pullulan extensively improved the thermal stability of CGPNH hydrogel when comparedwith cross-linked pullulan hydrogel (PNH).

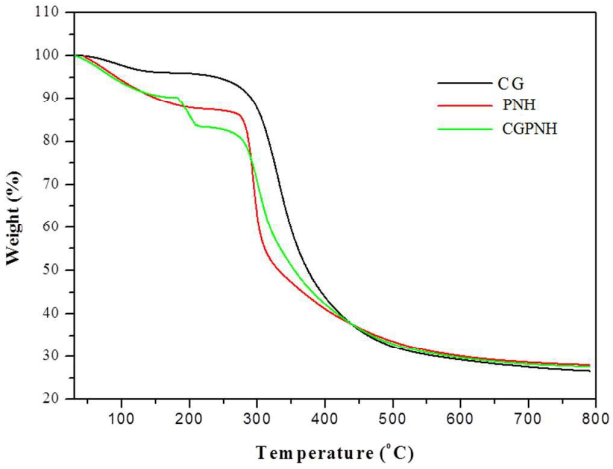

Fig. 5. TGA curve of fish skin collagen (CG), pullulan hydrogel (PNH) and collagen-pullulan hydrogel (CGPNH)

Differential Scanning Calorimetry (DSC)

Fig.6 illustrates the DSC thermograms of pure collagen, PNH and CGPNH hydrogels. Relatively large and sharp endothermic peaks observed in DSC curves for each of the membranes are due to their glass transition (Tg) of molecules at the particular temperature. Glass transition temperature of extracted fish skin collagen was found to be at ~ 89°C, whereas PNH hydrogel exhibit glass transition temperature at ~147°C, respectively[2,26,27]. The blended Collagen-pullulan hydrogel showed two thermogram peaks of glass transition temperature at 94 °C and 197° C. A possible cause of such a high glass transition temperature could be the existence of strong hydrogen bonding between the cross-linkedpullulan blended with collagen[25,27] (CGPNH).

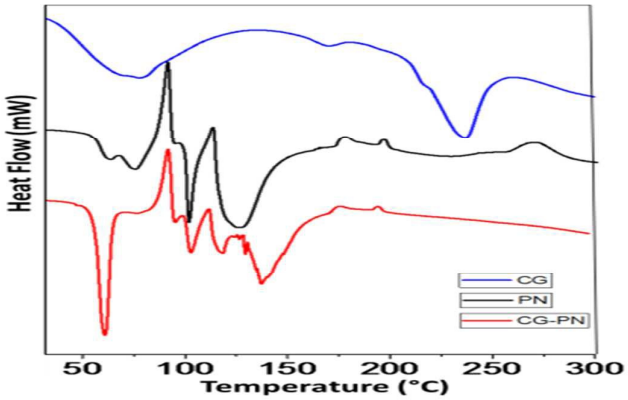

Fig. 6.DSC thermogram of fish skin collagen (CG), pullulan (PNH) and Collagen-pullulan hydrogel (CGPNH)

Fourier transform infrared spectroscopy (FTIR)

FTIR peaks of fish skin collagen (Fig.7) displayed bands at 1650, 1554 and 1253 cm^{-1}, which were the characteristics of amide I, II and III bands, respectively[28,29].The bands at 3421, 2925 cm^{-1} represent the stretching vibrations of N-H and C-H bonds, respectively[10,30]. The infrared spectra cross-linkedPNH hydrogel exhibited a strong absorption peak at 3315 cm^{-1}due to OH stretching, vibration and at 2940cm^{-1}due to the C-H stretching. It also displayed the typical absorption bands for α-configuration of α-d-glucopyranose units in pullulan at 849cm^{-1}. The FTIR spectra of the porous CGPNH hydrogel showed a broad peak around 3305cm^{-1} indicating stretching of hydroxyl groups and the absorption band at 3164cm^{-1}indicating the presence of a hydroxyl group with polymeric association[2,24,25]. However, the most characteristic bands of collagen were observed in the spectrum of hydrogel with two very sharp peaks at 1646cm^{-1}(N-H) and 1543cm^{-1}(C-O). At the same time, there was only a single peak at 1559cm^{-1}assigned to the stretching vibration of C-O in pullulan. Other features of the pullulan were also found from the spectrum, including C-H bend (1307 cm^{-1}), C-O stretch (1122 cm^{-1}) and very sharp peak at 1021 cm^{-1}belongs to the stretching of the C6-OH bonds. Moreover, the two main linkages of pullulan, α-(1,4) and α-(1,6)-d- glucosidic bonds were observed in the 741 $cm^{-1}$and 907 cm^{-1}. From the above discussion, confirms that the blending of collagen with pullulan has effectively occurred.

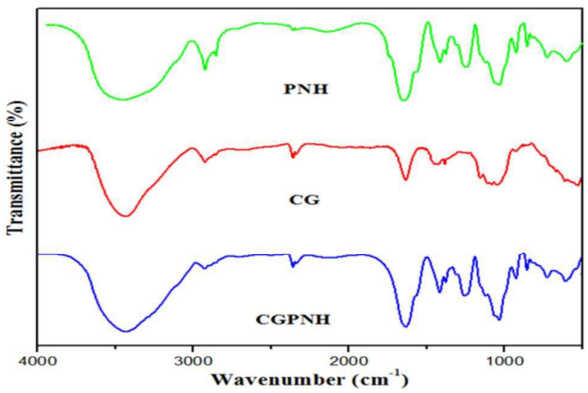

Fig. 7. FTIR analysis of pullulan hydrogel (PNH), fish skin collagen (CG) and Collagen-pullulan hydrogel (CGPNH)

Swelling studies

Swelling behaviour of fish skin collagen, cross-linked PNH and CGPNH hydrogels were investigated in PBS buffer at 37°C for 72h (Fig. 8). CGPNH hydrogel indicated highest water retention capacity (~390) than PNH hydrogel (~210%) and collagen (~110%). Swollen hydrogels retained their original shape and did not degrade after overnight incubation, due to these characteristic features it can be categorized as a superabsorbent. The water binding ability of the hydrogel membrane could be mainly attributed to the hydrophilic nature of the pullulan. In general, the water uptake decreases as the cross-linking degree is increases because of decrease in the number of hydrophilic groups as well as more difficulty in the structural expansion due to the more dense covalently linked network. This swelling property of the hydrogel combined with their porosity will greatly aid in the absorption of exudates in a wounded environment[27,31]. Hence the prepared CGPNH hydrogel may attribute an excellent wound dressing material.

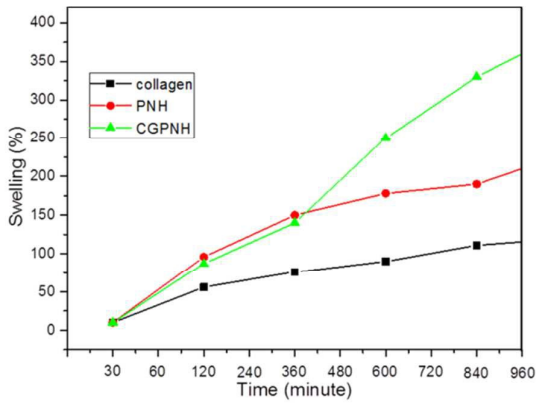

Fig 8. Swelling studies of fish skin collagen, PNH and CGPNH hydrogels

SEM Analysis

Fig.9.Showed SEM photographs of all the 5 samples of prepared hydrogel internal microstructure morphology. PNG and PNS hydrogel showed rough surface morphology whereas in CGPNH hydrogel produced more ordered porous structure with highly interconnected pores thanPNH hydrogel, which shows less porous structure.The CGPNH1 hydrogel showed irregular porous morphology, with increased pore size. It is assumed that these pores are the regions of water permeation and interaction sites of external stimuli with the hydrophilic groups of the hydrogels. Therefore the highly ordered porousstructure and relatively increased cross-linking density of the CGPNH hydrogels are found to be more suitablefor the application of wound dressing and targeted drug delivery applications.

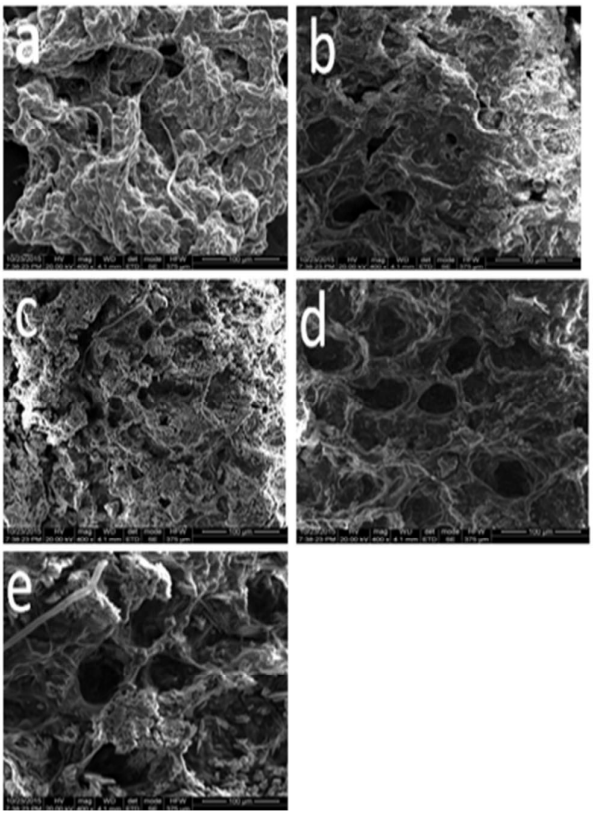

Fig. 9 SEM analysis ofprepared hydrogels surface morphology (a) -PNG, (b) - PNS, (c) – PNH, (d) – CGPNH, and (e) – CGPNH1 hydrogel. Among all the prepared hydrogels,the CGPNH hydrogel showed more ordered and highly interconnected porous structure.

In vitro biodegradation studies

The in vitro biodegradation studies of the hydrogel were assessed by measuring their degradation rate and biological stability against the collagenase A (2mg/ml), pullulanase (4U/ml) and the combination of both the enzymes (Fig.10). Collagenase brought about 64.2% and 37.9% weight loss while pullulanase showed 53.2% and 42.1% weight loss was observed in PNH and CGPNH samples over a period of 7 days. Whereas, the combination of these two enzymes degradation studies showed 80.2% and 31.7% weight loss in PNH and CGPNH, respectively. The resistance to enzymatic degradation of synthesized collagen blended pullulan hydrogel was greater than the cross-linked pullulan hydrogel. As expected, the CGPNH hydrogel showed improved mechanical property as well as reduced biodegradation rate.

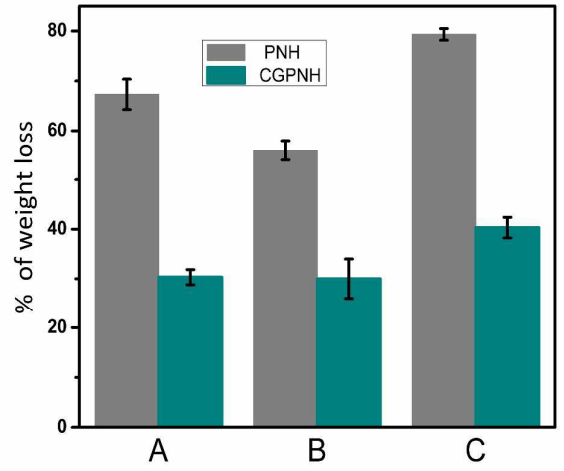

Fig.10. In vitro degradationstudies of CGPNH hydrogel and PNH hydrogels . (A)-collagenase, (B)-pullulanase and (C)-combination of collagenase and pullulanase enzyme. Data are presented as means ± SD (n = 3).

Heamocompatibility studies

The Heamocompatibility test results for Piscean collagen, PNH and CGPNH hydrogel were shown in Fig. 11. All the three samples were found to be below 2% haemolysis indicating that the prepared samples were highly hemocompatibleand suitable for biomedical applications.[32–34]

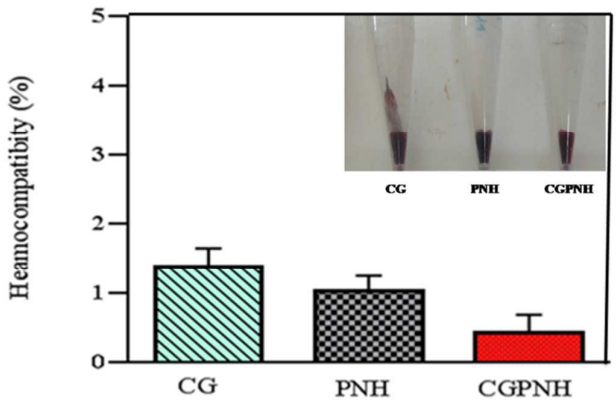

Fig 11. Hemocompatibility studies of CG, PNH and CGPNH hydrogels showing 100% heamocompatibility on the prepared samples.

CAM assay

Angiogenesis is a complex process involving the extensive interplay between cell soluble factors and extra cellular matrix (ECM) components[35]. Angiogenesis and neovascularization are critical determinants of the wound healing outcome. When compared withcontrol, CGPNH hydrogel promoted angiogenesis on chick chorioallantoic membrane on the 12th day (Fig.12). The newly formed blood vessel enhances the healing process by providing nutrition and oxygen to the growing tissues. It can be evident that the prepared CGPNH hydrogel has significantly promoted the growth of new blood vessel formation.

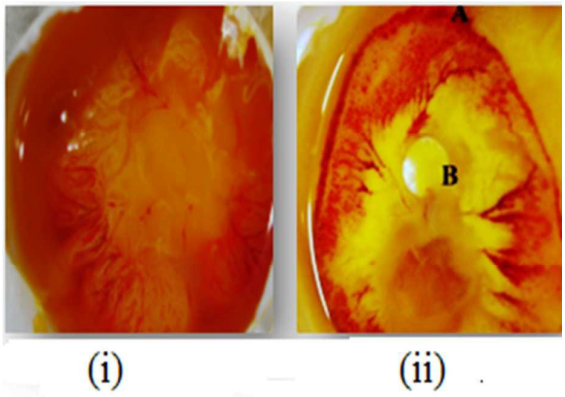

Fig .12. In vitro CAM assay (i) control (ii) CGPNH hydrogel, showed increased growth of blood vessel formation on chick chorioallantoic membrane on 12th day, when compared to control

Biocompatibility Studies (MTT assay)

Biocompatibility is known as the compatibility of the hydrogel with the immune system.MTT assay is used to demonstrate the biocompatibility of the hydrogel scaffolds (Fig.13). CGPNH enhances the growth and cell viability of the cells after 48h and 72h of incubation. Thus, the collagen-pullulan hydrogel significantly induces proliferation of NIH3T3 fibroblast cell lines, which was significantly ($p<0.05$) higher than that of PNH hydrogel.In the present study, cell adherence and cell proliferation potential of PNH and CGPNH hydrogels were assessed using NIH 3T3 cells. Fig.13 (a & b) illustrates the fluorescence image of the cells observed at different time intervals. The result showed that, no significant difference in fluorescence intensity in the synthesized CGPNH hydrogel treated cells, when compared to the control. Furthermore, no morphological changes were observed in the treated samples. Results of cell viability and quantification analysis showed 100% cell viability in both the samples suggested that the synthesized pullulan and collagen pullulan hydrogel do not have any negative impact on the cell viability. CGPNH hydrogel possesses an excellent 100% biocompatibility since their hydrophilic surface has a low interfacial free energy, may in contact with the body fluids which enhances the cells to adhere to the surface of the hydrogels. This excellent biocompatibility and proliferative potential of the hydrogel would definitely augment the healing process when used in a wounded environment.

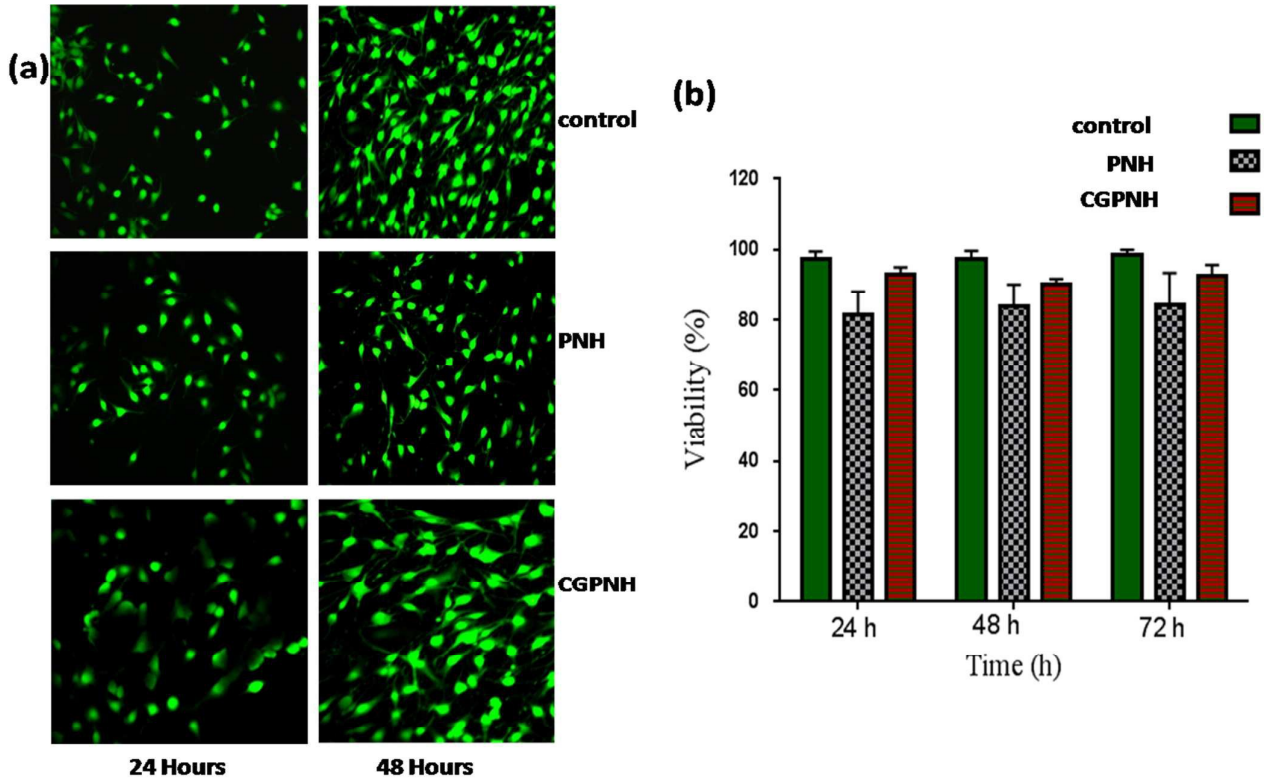

Fig 13. (a) In vitro cytotoxicity and cell proliferation studies ofthe prepared PNH and CGPNH hydrogels on NIH3T3 fibroblast cell lines for 48 hours were observed. **(b)**Biocompatabilityassessment of the samples using MTT assay by measuring the absorbance at 570nm in comparision with the control.The values are shown as means ± SEM *($P<0.05$).

Wound healing studies of hydrogel on animal models

Wound healing studies of hydrogel on the animal model was performed and healing of wounds covered with PNH hydrogel and CGPNH hydrogels was compared with an open wound (Fig.14). Healing rate of wounds treated with hydrogel dressing seems to proceed faster than untreated wounds. Histopathological evaluation of healing tissue on day 3, 9 and 12 (Fig.15) were studied. Hydrogel dressings adhered better to the wound bed and could be removed from the wound surface without causing any damage or trauma to the tissue. There was a marked infiltration of the inflammatory cells and increased blood vessel formation on CGPNH treated samples was observed on the 9th day studies, slightly improved progressive changes in inflammation with fibroblast growth was observed in PNH treated hydrogel whereas inflammation was still persistent in open wounds with the slow healing process. Full thickness epidermal regeneration which covered the wound area completely was achieved in day 12 observation with enhanced fibroblast proliferation, angiogenesis and re-epithelialisation in CGPNH treated wounds and for PHN treated wounds showed it took more than 14±2 days. Wounds with little inflammation and impaired healing were observed on day 12 in open wounds and for complete healing of the wound were achieved more than 19±2 days. On the basis of histopathological studies, it can be revealed that the prepared collagen blended pullulanhydrogenis highly potential in accelerating the wound healing process by providing a moist wound environment for rapid cellular repair and regeneration. Moreover, the superabsorbent nature of the hydrogel can absorb the excess exudates and prevent the microbial contact in wound bed also contributes rapid healing of the wound.

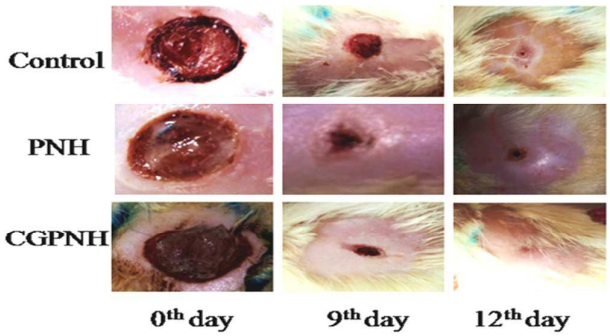

Fig. 14. Wound healing studies of hydrogels on excision wound on rats, showing CGNH hydrogels healed the wound faster (99%) than PNH (81%) and control (53%) on day 12.

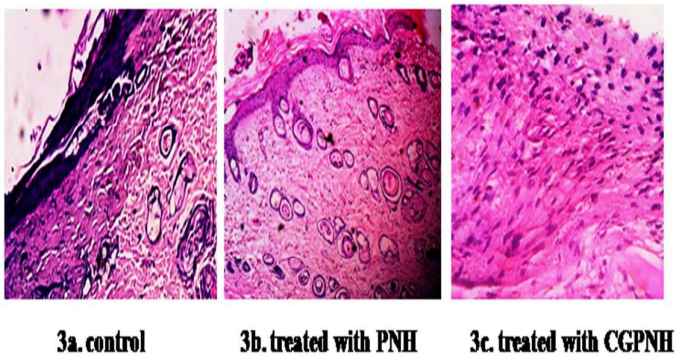

Fig.15. Histopathological studies on day 12 showed enhanced proliferation of CGPNH treated hydrogels than PNH and control.

Conclusion

The preparation of porous hydrogel from fish skin collagen and the polysaccharide pullulan was successfully fabricated and assessed its efficacy as a potential tissue engineering scaffold. The results revealed that the collagen blended with pullulan has significantly improved thermal stability and mechanical properties with excellent hydrophilicity and biocompatibility. Wound healing studies showed that these hydrogels can absorb the exudates and reduce the trauma by maintaining moist wound healing environment. It also promotes wound healing by enhancing granulation, tissue regeneration and formation of new blood vessels in the wounded sites. The combination of the prepared hydrogel from the natural polymers alone can act as a potent wound dressing material without adding any drug; this will also add advantages include simplicity, versatility, non-toxic and cost effective. Since the results are promising, further experiments could be tried to enable its use in clinical applications.

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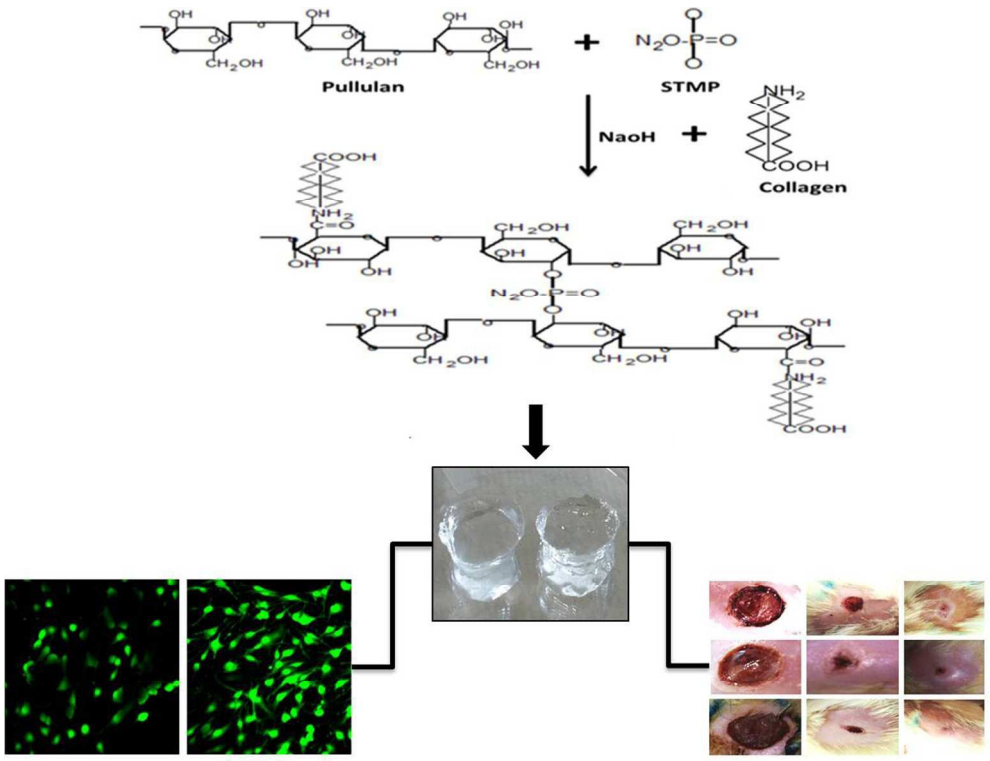

Synthesis of collagen- pullulan hydrogel for skin tissue engineering

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