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Chitosan-Chondroitin sulfate based polyelectrolyte complex for effective management of chronic wounds

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ABSTRACT

Acute and chronic wound remain an unresolved clinical problem among various demographic groups. Traditional marketed products focus mainly on inhibition of bacterial growth at the wound site neglecting the tissue repair, which significantly affect the healing rate. It would be highly beneficial if a wound healing material can be developed which has both antibacterial as well as tissue regenerating potential. We have prepared a polyelectrolyte complex (PEC) using chitosan (CH) and chondroitin sulfate (CS) which can form an in-situ scaffold by spontaneous mixing. The fabrication of CH-CS PEC was optimized using Quality-By-Design (QbD) approach. The prepared PEC showed very high swelling and porosity property. It was found to be non-hemolytic with good blood compatibility and low blood clotting index. It also exhibited good antibacterial activity against both gram-positive and gram-negative bacteria. The cell proliferation study exhibited good cytocompatibility and almost four-fold increase in cell density when treated with CH-CS PEC compared to control. In summary, we demonstrated that the prepared CH-CS PEC showed good blood compatibility, high antibacterial effect, and promoted wound healing potentially by stimulating fibroblast growth, making it an ideal wound dressing material.

1. Introduction

Skin is the largest organ of the human body which acts as a protective barrier against microbial invasions and maintains body's physiological homeostasis [1]. It can get damaged by several external factors including burn, physical injury, and different disease states like diabetic ulcer etc. Acute and chronic wound is an unresolved clinical problem among various demographic groups. 1% to 2% of the total population in developing countries is estimated to experience a chronic wound during their lifetime [2]. An ideal wound dressing materials should act as a dermal substitute at the wound site with optimum porosity for gaseous exchange, possess biological properties like supporting cell adhesion and cell proliferation, provides a moist environment and should have antibacterial property [3]. Along with that, the dressing should be non-toxic, non-allergenic and should be made of readily available biocompatible materials with ease of manufacturing. So far, several kinds of wound dressings have been developed including polymeric scaffolds, hydrogels, films, gauge, foams, membranes etc. [4]. Role of natural polymers in wound care is well documented due to its biocompatibility with the dermal tissues and biodegradative property [5]. Biopolymers like chitosan, hyaluronic acid, collagen, alginic acid etc. are widely used in wound management [6]. Among these natural polymers, chitosan (CH) is one of the most widely explored for wound healing purpose due to its beneficial properties such as biodegradability, biocompatibility, antibacterial and hemostatic property [7]. CH is a linear cationic polysaccharide composed of randomly distributed β-linked D-glucosamine and *N*-acetyl-D-glucosamine.

Many different CH based wound healing material has been marketed including HemCon®, Syvek-Patch®, Chitopack C®, Beschitin®, Tegasorb® 3 M, Chitodine® IMS etc. [3]. However, a major limitation of using CH as wound healing material is its inadequate mechanical strength. The scaffold prepared with only CH has high brittleness and henceforth its application is compromised as wound dressing material [8]. To improve the mechanical property of CH, different strategies have been adopted, including chemical modification of CH [9], mixing other polymers with CH [10] or making non-covalent complexes of CH with other molecules [11]. Among these strategies, the formation of polyelectrolyte complex (PEC) is an interesting approach to improve the mechanical property of CH and also to impart other beneficial features in the composite material [12]. PECs are the association complexes formed between oppositely charged polyelectrolytes (e.g. polymer-polymer, polymer-drug and polymer-drug-polymer). Stoichiometric combinations of the polycation and polyanionic compounds can result in formation of PEC [13]. Generally, oppositely charged polyions (polycations or polyanions) undergo electrostatic interactions between them resulting in the formation of PEC. The advantage with PEC is that they can be formed without the use of chemical cross-linking agents, thereby making the synthesis process very simple and straightforward.

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As CH acts as a polycation due to the presence free -NH_2 group in the D-glucosamine units, CH acts as a polycation. Different types of PECs have been designed using CH as the cationic counterpart with diverse types of polyanions [12]. Few examples of CH based PECs for wound healing application is also there. Vasile et al. prepared a CH and hyaluronic acid PEC for the treatment of burn wounds [14]. Kim et al. developed a CH and sodium alginate based PEC for wound dressing [15]. Similar CH/alginate PEC has also been reported by Hong et al. [16]. Puppi et al. prepared Chitosan/poly(γ-glutamic acid) polyelectrolyte complex based 3D scaffold for tissue engineering [17].

The main disadvantage associated with most of these PECs is that as they are composed of two high molecular weight polymers (CH/hyaluronic acid; CH/sodium alginate; etc.), their spontaneous mixing is not possible. Hence, the wound healing scaffolds need to be prefabricated for the application. However, if a low molecular weight polyanion is used, spontaneous mixing can occur, leading to the formation of a PEC in-situ. The major advantage of spontaneous PEC formation is size and shape modulation which can be done relatively easily with this method. Structurally, a wound healing scaffold can be of mainly two types, amorphous or semi-stiff sheets. Amorphous scaffolds have very low mechanical strength and can be packaged into a tube for ease of handling. It can also conform to the irregular depths of a wound bed. However, the porosity of amorphous scaffolds is significantly low, hence it cannot absorb high amount of tissue exudates. Also, due to the lower mechanical strength of amorphous scaffolds, it cannot act as a basement membrane to stimulate dermal tissue proliferation. Contrarily, semi-stiff sheets that have enough structural strength have good porosity and absorption capacity and can stimulate dermal tissue growth by acting as a membrane. However, handling of these sheets is difficult as they come pre-fabricated and needs special packaging and handling. They also cannot take-up the shape of the wound due to their stiffness. It would be highly beneficial if we can develop a scaffold that has the property of both types, i.e., it should be amorphous in nature for ease of application, storage and handling, yet, after application, will have sufficient mechanical strength to act as a scaffolding material to improve tissue repair and absorb wound exudates.

In the current study, we have selected chondroitin sulfate (CS) as a polyanion to be used with CH to make a PEC scaffold. CS is an unbranched oligosaccharide containing two alternating monosaccharides: D-glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine (GalNAc). CS has a wide range of bioactivity including promoting tissue regeneration, intracellular signaling, cell proliferation, and cell adhesion [18]. Due to the presence of glucuronic acid moiety, CS has an overall negative charge. In the current study, we have used a disaccharide form of CS with molecular weight of 475 Da. Due to its low molecular weight, CS solution has a very low viscosity and can freely diffuse through a high viscosity CH polymer solution. As high molecular weight CH solution makes a gel type structure, if CS solution is applied on top of this CH gel, it would percolate into the gel and make electrostatic interactions with the CH polymer, creating a PEC scaffold in-situ.

Preparation of PEC using different varieties of CH and CS has been reported for many different purposes ranging from drug delivery to tissue engineering and regeneration [19–29]. However, till now, no one has explored this spontaneous mixing of CH and CS for making an in-situ scaffold for use in wound healing. In the current study, we first prepared the scaffold of CH-CS-PEC without the use of any catalyst and crosslinkers with simplicity in the method of preparation. Then we have performed detailed physicochemical characterization of the scaffold to establish formation of a complex between these two polymers. Then the composition of the PEC was optimized for best wound healing property by varying different parameters like concentration of each polymer, the molecular weight of polymer etc. and the optimized composition was validated using Quality-By-Design (QbD) approach. As chitosan is considered as a standard wound care polymer, different physicochemical properties like hemostatic potential and thrombotic effect of the CH-CS-PEC was evaluated and compared with scaffold prepared with only CH. Finally, we have done antibacterial and cell proliferation study to establish the effectiveness of the CH-CS-PEC for wound healing purpose.

2. Experimental

2.1. Materials

High molecular weight CH (MW ≈ 700 kDa, DD: min 90%), medium molecular weight CH (MW ≈ 310 kDa, DD: min 90%) and low molecular weight CH (MW ≈ 190 kDa, DD: min 90%) was purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Maharastra, India). Chondroitin 6-sulfate (PubChem CID: 24766) mono sodium salt, MW 475.39 Da was procured from Tokyo Chemical Industry Co. Ltd. Ninhydrin and Bovine serum albumin were procured from S.D. fine chemicals Ltd. (Maharastra, India). Triphenyl tetrazolium dye was obtained from Sigma-Aldrich, India. L929 human dermal fibroblast cells were obtained from NCCS, Pune. Dulbecco modified Eagle medium, fetal bovine serum was obtained from HiMedia laboratories Ltd. DAPI- mounting media was purchased from Thermo scientific, India. All other chemical reagents used were of A.R. grade.

2.2. Preparation of CH-CS PEC

Solutions of polymers were prepared separately by solubilizing CH in 1% w/v glacial acetic acid solution and CS in distilled water. CH solution was then dialyzed using a 10 kDa cut-off membrane against MQ water to remove the acetic acid. Dialysis was stopped when the pH of the CH solution becomes more than or equal to 6. For sterilization, CH solution was sterilized by autoclaving whereas CS solution was sterilized by filtration through a 0.22 μm membrane filter. CH-CS PEC was fabricated by mixing both the polymers in 1:1 v/v ratio. Briefly, highly viscous CH solution was poured on the mold initially. On top of that, an equal quantity of CS solution was added drop-wise. After few seconds, formation of an opaque cross-linked scaffold was observed which confirms the formation of polyelectrolyte complex. For the physicochemical evaluation of the prepared CH-CS PEC scaffold, the prepared PEC was lyophilized for 24 h to make it dry.

To compare the proposed CH-CS PEC with that of CH, scaffold with only CH has also been prepared. CH scaffold was prepared by making a solution of CH in the same way as described above and after dialysis to remove acetic acid, a measured amount of the CH solution was poured on the mold. The solution was frozen and lyophilized to make it dry. In this scaffold, the same CH at the same concentration as that of CH-CS PEC was used to determine the effect of only CH on the physicochemical properties of the scaffold.

The formulation was optimized exploring Quality-By-Design approach using 3^3 Box Behnken Design (BBD). The experimental design matrix was prepared and run in Stat-Ease Design-Expert version.8.0.0.7. software. The experiments were run as per the design matrix obtained by the software. The critical quality attributes (CQA) were selected as the concentration of CH (X_1), the concentration of CS (X_2), and molecular weight of CH (X_3). The quality target product profile (QTPP) was chosen as per the formulation requirement of the ideal wound dressing, namely % swelling and % porosity of the prepared CH-CAS PEC scaffold. Table 1 shows the design matrix obtained by using BBD design. Table 2 describes the design matrix of the 3^3 Box Behnken design obtained through Design expert software.

2.3. Characterization of CH-CS PEC complex

2.3.1. Fourier transform infrared spectroscopy

FTIR spectroscopy was performed to confirm the formation of CH-CS PEC scaffold. Fourier transform infrared spectroscopy was done using IR

Table 1
Experimental design, factors, and responses.

Independent variables	Levels		
	−1	0	+1
X_1 = Concentration of CH (%)	2	5	8
X_2 = Concentration of CS (%)	2	5	8
X_3 = mol. wt of CH (KDa)	Low	Medium	High
Dependent variables			
Y1 = Swelling study			
Y2 = Porosity			

Prestige FTIR instrument (Shimadzu). CH-CS PEC was dried by lyophilization and the dry powder used for FTIR analysis. The samples were prepared using KBr pressed pellet method in 1:100 ratio of the sample and KBr. The sample was analyzed in between 400 and 4000 cm^{-1}. FTIR spectroscopy of CH, CS and the CH-CS PEC was done separately.

2.3.2. Differential scanning calorimetry

Differential scanning calorimetry measurements were performed in a DSC-60 plus thermal analyzer (Shimadzu). The DSC curves were executed under a dynamic nitrogen atmosphere (40 ml min^{-1}) using different sample mass as 2–5 mg and heating rates 10 °C min^{-1}. Accurately weighed samples were placed into a covered aluminum sample holder. Empty aluminum sample holder was kept as a reference and the run was performed by heating the samples from 25 °C up to 480 °C.

2.3.3. Swelling studies

The swelling index was calculated by the method reported by Im et al. [30]. Briefly, pre-weighed CH-CS PEC scaffold was immersed in PBS (pH 7.4). The weight of the prepared PEC was calculated at different time points till 5 h. Swelling ratio was calculated by:

$$\text{Swelling ratio} = ((W_w - W_i)/W_i) \times 100$$

where W_i was the initial weight of the sample and W_w was the wet weight of the samples at the respective time interval.

2.3.4. Porosity studies

The porosity studies were performed by alcohol displacement method. Pre-weighed CH-CS PEC scaffold was dipped in a graduated cylinder containing ethanol and soaked for 24 h. The final weight of the wet sample was noted as W_f % porosity was determined using formula reported by Im et al. [30].

$$\text{Porosity}\% = ((W_f - W_i/\rho_{ethanol})/V) \times 100$$

W_f and W_i indicates the weight of samples before and after immersion in alcohol, respectively. V is the volume of alcohol used in the study ρ ethanol is the density of ethanol.

2.3.5. Scanning electron microscopy

The surface morphology of the CH-CS PEC was studied using the scanning electron microscopy. SEM images of the CH-CS PEC scaffold was taken at high vacuum mode in a Apreo-S FEI instrument at 20 μm scale for observing the morphology of the CH-CS PEC. The images of scaffold prepared with the different molecular weight of CH and scaffold prepared with CH alone were also captured for comparison purpose.

2.3.6. Degradation studies

As the CH is rich with the amino group, the degradation profile of the CH-CS PEC was analyzed using ninhydrin reagent. Ninhydrin reagent reacts with the amine group and produces a deep blue color product, which can be quantified at 570 nm. The hypothesis is, when the CH-CS PEC undergoes degradation, free CH will be released from the scaffold, which can then be quantified using ninhydrin reagent. Slandered curve for quantification was prepared with free CH. The study was performed at different pH 4, pH 7 and at pH 9. The preweighed CH-CS PEC scaffold was immersed into the buffer having pH 4, pH 7 and pH 9. At respective time interval, the samples were aliquoted and sink condition was maintained. To the 1 ml of the aliquoted samples an equal volume of 8% ninhydrin solution was added and the solution was heated at 80 °C for 15 min. The solution was allowed to cool and was analyzed at 570 nm using UV spectroscopy.

2.4. In-vitro hemolysis test

In-vitro hemolysis was studied to check the hemocompatibility of the prepared CH-CS PEC. The hemolysis ratio of CH-CS PEC scaffold was tested with different concentration of CH-CS PEC (2 mg/ml to 0.008 mg/ml). The samples were preweighed in the required quantity and were UV sterilized for 30 min. The samples were crushed in a mortar and pestle and the required concentrations were prepared by suspending the sample in PBS. The blood was withdrawn from Wistar rat via the retro-orbital route. To prevent it from clotting, it was mixed with the required amount of 0.1 M EDTA (20 μl/ml blood). The blood sample was then centrifuged at 7500 rpm twice for 7 min to isolate RBCs. The supernatant was removed and the RBC pellet was washed with PBS (pH 7.4). Finally, the RBCs were suspended in 1 ml PBS (pH 7.4). From the suspension of RBC 100 μl of sample was added to different concentration of CH-CS PEC. The blood sample mixed with distilled water was taken as a negative control. The samples were incubated for 1 h with gentle shaking. The samples were then again centrifuged at 3500 rpm for 1 min to remove intact RBCs. The supernatant was isolated and analyzed using UV spectrophotometer at 408 nm. The % hemolysis was calculated using the formula:

$$\%\text{Hemolysis} = \left(\frac{Ds - Dn}{Dp - Dn}\right) \times 100$$

where Ds, Dn and Dp are the absorbance of the sample, normal saline mixed with samples, and the distilled water mixed with samples respectively.

Table 2
Composition of experimental formulations.

Batches	Factor		
	X_1	X_2	X_3
	Concentration of CH	Concentration of CS	MW of CH
1	5	8	High
2	8	5	High
3	2	5	High
4	5	2	High
5	5	5	Medium
6	8	2	Medium
7	8	8	Medium
8	2	2	Medium
9	2	8	Medium
10	5	2	Low
11	5	8	Low
12	8	5	Low
13	2	5	Low

2.5. Whole blood clotting study and cell adhesion test

The whole blood clotting study was performed to check the hemostatic potential of the CH-CS PEC scaffold. The smaller the blood clotting index is, the stronger the hemostatic potential of the material. The blood was collected via retro orbital route from the wistar rat. The study was performed by putting 0.2 ml citrated whole blood onto the preweighed scaffold. On top of the treated scaffold, 20 μl of 0.2 M $CaCl_2$ solution was added to initiate coagulation. After 10 min, red blood cells (RBCs) that were not trapped in the clot were hemolyzed with 20 ml of deionized water, and the absorbance of the resulting hemoglobin solution was measured at 408 nm. Blood sample without CH-CS PEC was taken as blank. The study on blood clotting mainly evaluates the hemostatic potential of the wound dressing to induce thrombosis in blood by a performance evaluation parameter called blood-clotting index (BCI). The blood cells trapped in the CH-CS PEC were fixed by treating the treated sample with 4% glutaraldehyde solution for 15 min. The sample was then dried and its morphology was studied using scanning electron microscopy (Apreo-S FEI) to check the amount of the RBCs adhered on the surface of CH-CS PEC. Blood clotting index was calculated using the formula:

$$BCI = As/Ab \times 100$$

where A_s is the Abs of the sample and A_b is the Abs of blank [31].

2.6. Protein adsorption study

The adsorption of bovine serum albumin (BSA) was done via batch contact method [32]. Increase in adsorption of bovine serum albumin implies better thrombotic property. The samples were first soaked in 20 ml of 0.2 wt% BSA solution in PBS (pH = 7.4) and then were shaken for 1 h to prevent the solution-air interface formation. The supernatants were analyzed to determine the residual BSA in solution using ultraviolet spectrophotometer at 280 nm. The amount of protein adsorbed on the CH-CS PEC was calculated using the formula:

$$\text{Adsorbed BSA (mg/g)} = (((C_o - C_a)/w) \times V)$$

where C_0 and C_a are the BSA concentrations (mg/ml) before and after adsorption, respectively, w is the weight of the swollen CH-CS PEC (g) and V is the volume of the BSA solution (ml).

2.7. Antibacterial activity

The study was performed using micro-broth dilution method using tetraphenyl tetrazolium chloride (TTC) dye. The experiment was performed on both gram-positive (*Bacillus subtilus*) and gram-negative (*Escherichia coli*-DH5α) strains. The study was performed using CH-CS PEC, CH and CS. Microbial inoculums were prepared by subculturing microorganisms into Luria Bretani (LB) broth at 37 °C for 18 h and were diluted to approximately 10^5 to 10^6 of organisms/ml in two-fold LB broth. A stock solution of the 2 mg/ml of CH-CS PEC, CH and CS were prepared in growth media i.e. LB broth. Further serial dilutions were made in the range of 2 mg/ml to 0.125 mg/ml of both the formulations. A 100 μl aliquot of each dilution was added to 100 μl LB broth containing each test microorganism and was added to the 96 wells microplate. The microplates were then incubated at 37 °C for 24 h. After incubation, 40 μl of Triphenyl tetrazolium chloride (1.0 mg/ml, TTC) was added to each well and was incubated for 3 h. Color changes of TTC in the microplate from colorless to red were accepted as microbial cell viability and intensity was observed at 450 nm. [33]

2.8. Cell proliferation assay

In-vitro cell proliferation study was conducted on L929 mouse dermal fibroblast cells. The L929 fibroblast cells were grown and maintained in Dulbecco Modified Eagle Medium with 10% FBS. In-vitro cell proliferation assay was studied to check the rate of proliferation of the L929 dermal fibroblast cells with CH-CS PEC. For the study, first glass cover-slips were coated with the CH scaffold and CH-CS PEC. Briefly, a measured amount of CH solution was added on the cover-slip and it was frozen and lyophilized. For CH-CS PEC, an equal amount of CH and CS solution was added sequentially on the cover-slip to make the PEC, which was subsequently frozen and lyophilized. Both the scaffolds were sterilized by UV exposure. The cells were then seeded on the scaffold coated cover-slips. Five different groups were taken, namely CH-CS PEC, CH scaffold, CS solution, CH scaffold+CS solution and control. For CS solution, cells were grown on normal glass cover-slip on which CS solution treatment was given. As CS is of low molecular weight and does not make a scaffold on its own, we treated it like this. For CH scaffold+CS solution, cells were first seeded on the CH scaffold coated cover-slip which was then treated with CS solution. Control cells were grown on glass cover-slip without any treatment. The cell proliferation was examined at 12 h, 24 h and 48 h under a fluorescent microscope. Cells were fixed with 1 ml of 100% methanol and were mounted with DAPI stain. The cell proliferation in the treated samples was observed using a fluorescent microscope (Zeiss). All the samples were observed at 10× magnification. The cell count for each sample was done using Image J software.

2.9. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was conducted with the two-tailed unpaired *t*-test for two-group comparison or one-way ANOVA, followed by the Tukey multiple comparison tests by using GraphPad Prism (for three or more groups). A difference with $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Design and fabrication of CH-CS PEC

CH is a linear polysaccharide composed of randomly distributed β-(1 → 4)-linked D-glucosamine. Due to the presence of the free —NH_2 group in the glucosamine unit, CH can readily get protonated in the acidic medium and carry a positive charge. CS, on the other hand, is an oligosaccharide and composed of a chain of alternating sugars, sulfated *N*-acetylgalactosamine and glucuronic acid. CS carries negative charge due to the presence of sulfate group and glucuronic acid moiety. As CH and CS carry opposite charges, these two polysaccharides can spontaneously interact to form a polyelectrolyte complex (PEC). The advantages with PEC include quick and spontaneous formation, stability, etc. [34]. Along with these, CH and CS have the added advantage of spontaneous mixing. We have used a disaccharide form of CS (6 sulfate, monosodium salt) in this study with molecular weight of 475 Da. As CH has significantly higher molecular weight than CS, CH solution is highly viscous whereas CS solution of same concentration has very low viscosity. Due to this, CS solution can easily percolate into the viscous CH solution, making cross-links on the way, forming the PEC leading to instant scaffold formation (Fig. 1), while with other PEC formation, the polymer solutions need to be mechanically mixed. CH solution when poured into a Petri dish, formed a thick and viscous layer. We added CS solution on top of it, which easily penetrated into the CH solution, forming the CH-CS-PEC as a solid scaffold rapidly (Fig. 1). This spontaneous formation is highly beneficial over prefabricated scaffolds used for wound healing as size and shape modulation can be done relatively easily with this method. However, the scaffold prepared by spontaneous mixing of two polymer solutions may not be uniformly homogeneous, due to the uneven mixing of the polymers. This could be considered as a disadvantage of this process.

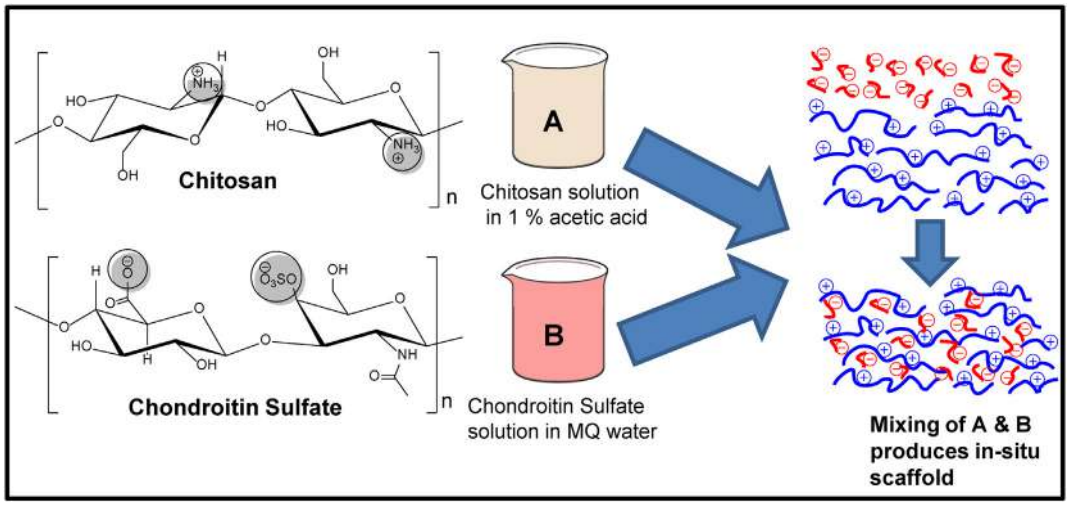

Fig. 1. Schematic diagram depicting the mechanism for formation of the CH-CS PEC.

Few researchers have previously prepared CH-CS PEC for diverse application. For example, Huang et al. have reported CH-CS PEC microcapsules for controlled release of 5-fluorouracil [28]. They prepared the microcapsules by emulsion-chemical crosslinking method. Sui et al. have also reported a similar microcapsule, using the same emulsion-chemical crosslink method [29]. Yeh et al. have prepared protein-loaded CH-CS PEC nanoparticles by ionic cross-linking of CS solution with CH [27]. When they mix dilute solutions of CH and CS, NP suspension was formed. Similarly, Umerska et al. prepared calcitonin loaded CH-CS PEC nanoparticles by ionic cross-linking method [26]. Similar CH-CS PEC nanoparticles were prepared by Tan et al. [20] and Jardim et al. [22] as well, encapsulating anthocyanins and curcumin respectively. Fan et al. have reported preparation of a covalently linked CH-CS hydrogel, using Schiff' base reaction between amino and aldehyde groups of polysaccharides for crosslinking [21]. Alinejad et al. have prepared an injectable PEC hydrogel using CH solution mixed with the beta-glycerophosphate/sodium hydrogen carbonate along with CS solution [19]. The solutions were mixed thoroughly using two syringes and a Luer Lock connector to make the hydrogel. Park et al. have prepared a porous CH-CS sponge by freeze drying and crosslinking CH and CS with tripolyphosphate [24]. Recently, Concha et al. have reported preparation of an aerogel using CH and CS [25]. For developing the aerogel, they first prepared a colloidal suspension of CH-CS PEC and freeze-dried it to get the aerogel. However, till now, no one has explored spontaneous mixing of high molecular weight CH and low molecular weight CS for making an in-situ scaffold for use in wound healing.

3.2. Physicochemical characterization of the CH-CS-PEC

3.2.1. FT-IR spectroscopic analysis

After fabricating the scaffold, we wanted to verify whether a PEC is forming between CH and CS or it's only a physical mixture. For that, FTIR studies were conducted with the powder prepared from dried (lyophilized) CH-CS PEC. Fig. 2A describes the FTIR spectra of CH, CS and the CH-CS-PEC prepared with high molecular weight chitosan and chondroitin sulfate. The peak for —NH_2 group bending and stretching, appeared around 1642 cm^{-1} and 3486 cm^{-1}, respectively, present in the FT-IR spectra of the CH. However, it disappeared in the CH-CS-PEC, indicating modification in the —NH_2 group. The intensity of the peak assigned to the negatively charged —OSO^{3-} group in the CS, which was coming at 1217 cm^{-1}, was reduced in the CH-CS-PEC. The carbonyl carbon (C═O) peak of CS at 1650 cm^{-1} also shifted and reduced in intensity in the CH-CS-PEC. These data confirm the ionic bond formation between the positively charged amino group of CH and the negatively charged sulfate and the carbonyl group of CS.

3.2.2. DSC analysis

Next, we confirmed formation of the CH-CS-PEC with DSC thermogram analysis. This study was also done with the powder prepared from dried (lyophilized) CH-CS PEC. As depicted in Fig. 2B, native CH exhibited an endothermic peak at ~70 °C and an exothermic peak at ~300 °C. CS exhibited an endothermic peak at ~93 °C and a sharp exothermic peak at ~240 °C. Compared to that, with the CH-CS-PEC, a completely new endothermic peak was detected at ~62 °C and two exothermic peaks at ~308 °C and ~460 °C (Fig. 2B). This data indicates that the complex formation was successful.

3.3. Optimization of the CH-CS-PEC design based on swelling and porosity analysis

After confirming that CH and CS can spontaneously interact to produce a PEC, we wanted to determine the best ratio of CH and CS to produce the most useful scaffold to be used for wound healing purpose. To find out the best ratio of CH and CS, Box Behnken design was chosen for the optimization of CH-CS-PEC. Based on our preliminary experiment and understanding, three independent variables were chosen for optimization, concentration of CH, concentration of CS and MW of CH. Three different concentrations of CH and CS (2, 5 and 8 wt%) and three different MW of CH (low, medium and high) were selected, as depicted in Table 1. These variables were used with Design Expert software (version 8.0.0.7) to construct the design matrix (Table 2). Based on this design matrix, PEC was prepared and tested for their efficacy. We have chosen swelling and porosity as the quality target product profile (QTPP) as these two properties are essential requirements of a wound dressing material. Higher swelling and porosity property defines that the dressing will absorb wound exudates, promoting wound healing. The swelling and porosity of different batches of PEC are depicted in Table 3 and Fig. 3A and B. Among the three variables, MW of CH was found to have the most significant impact on the QTPP of the CH-CS-PEC and high MW showed enhanced swelling and porosity compared to the low and medium MW CH (Fig. 3A, B, Table 3). This may be due to the longer chain length of the high MW CH, which increases the entanglement of the polymer, increasing porosity. The effect of MW of polymer on the porosity of PEC has been studied before and a similar phenomenon has been observed [35]. Other two variables, namely concentration of CH and concentration of CS, were found to have a moderate effect on the QTPP, and higher concentration of both CH and CS generally increased the QTPP slightly. We also found that >5 wt% of high MW CH was very difficult to dissolve, and with an increase in the concentration of CS beyond 5 wt%, though there was a slight increase in swelling, there was a decrease in porosity.

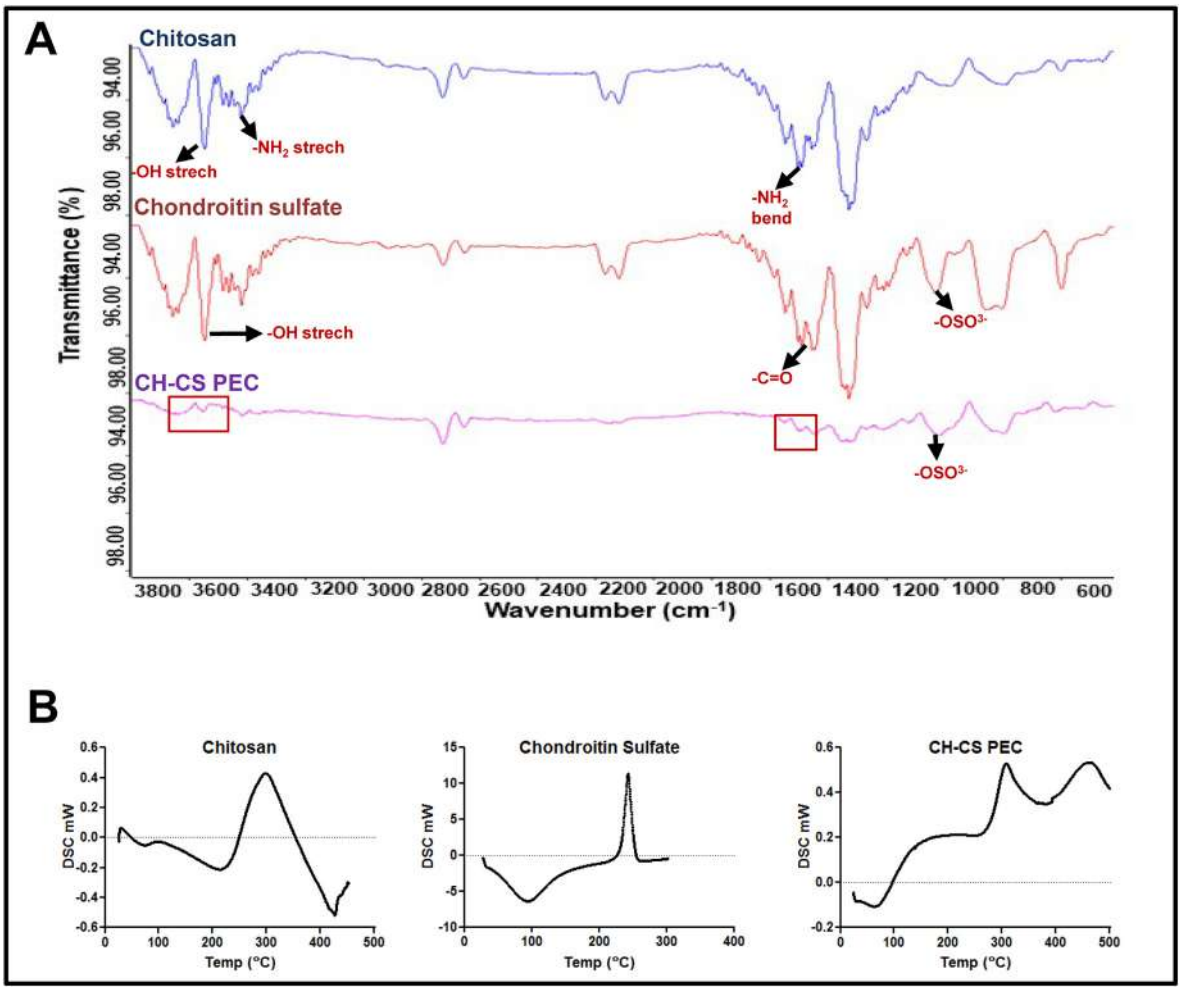

Fig. 2. Physicochemical characterization of the CH-CS-PEC. A. FT-IR spectroscopic analysis of CH, CS and CH-CS PEC; B. DSC analysis of CH, CS and CH-CS PEC.

3.4. Influence of independent variables on the swelling property of CH-CS-PEC

Next, we analyzed the effect of the independent variables on the swelling property and porosity of the CH-CS-PEC. As higher swelling property was found with high MW CH (X_3), it was kept constant. The 3-D contour plot (Fig. 3C) describes the effect of changes in concentrations of both the polymers on % swelling. It extrapolated that as the concentration of CH and CS increased, the % swelling of the polyelectrolyte complex was also increased. The quadratic model was considered to be the best-fit model for the swelling property of the polyelectrolyte complex with p-value 0.0369. The model followed polynomial equation $Y = +155.67X_1 - 69.12X_2 + 586.09X_3 - 17.55X_1^2 + 8.59X_2^2 + 133.27X_3^2 - 2.72X_1X_2 - 78.82X_1X_3 + 6.104X_2X_3$. The equation determines that variables concentration of CH (X_1) and MW of CH (X_3) has a significant impact on the swelling property of CH-CS PEC.

Fig. 3D depicts the 3D contour plot of the effect of independent variables on the % porosity of CH-CS PEC. It was found that there was no significant impact of the change in concentration of CS on the porosity of the CH-CS PEC, and henceforth was kept constant. The other two factors i.e. concentration of CH and molecular weight of CH showed a significant impact on the % porosity of the CH-CS PEC. The best model fit for % porosity was a linear model. The contour plot described the positive impact of MW of CH on the porosity property of the PEC. The graph determined increase in porosity with increase in concentration and molecular weight of CH. The polynomial equation defining the model is $y = +53.12 - 5.12X_1 - 5.29X_2 + 52.25X_3$. The p-value for the model was 0.0136 and the lack of fit value was 7.86.

Table 3
Quality target product profile of CH-CS PEC.

Batches	Factor			Responses	
	X_1	X_2	X_3	Swelling study	Porosity
	Concentration of CH	Concentration of CS	MW of CH	(%)	(%)
1	5	8	High	928.98	104.2
2	8	5	High	Batch didn't form	
3	2	5	High	879.49	81.49
4	5	2	High	865.12	108.65
5	5	5	Medium	345.05	56.79
6	8	2	Medium	385.23	65.06
7	8	8	Medium	232.23	70.18
8	2	2	Medium	247.63	76.77
9	2	8	Medium	278.00	20.76
10	5	2	Low	219.06	22.45
11	5	8	Low	234.67	28.84
12	8	5	Low	235.71	20.42
13	2	5	Low	167.76	18.09

3.5. Validation of the Design Analysis approach

To validate the use of the Design Analysis approach for determination of the best composition, some combinations of checkpoint were generated by the software predicting the range of independent variables and the dependent variables. Three different batches were

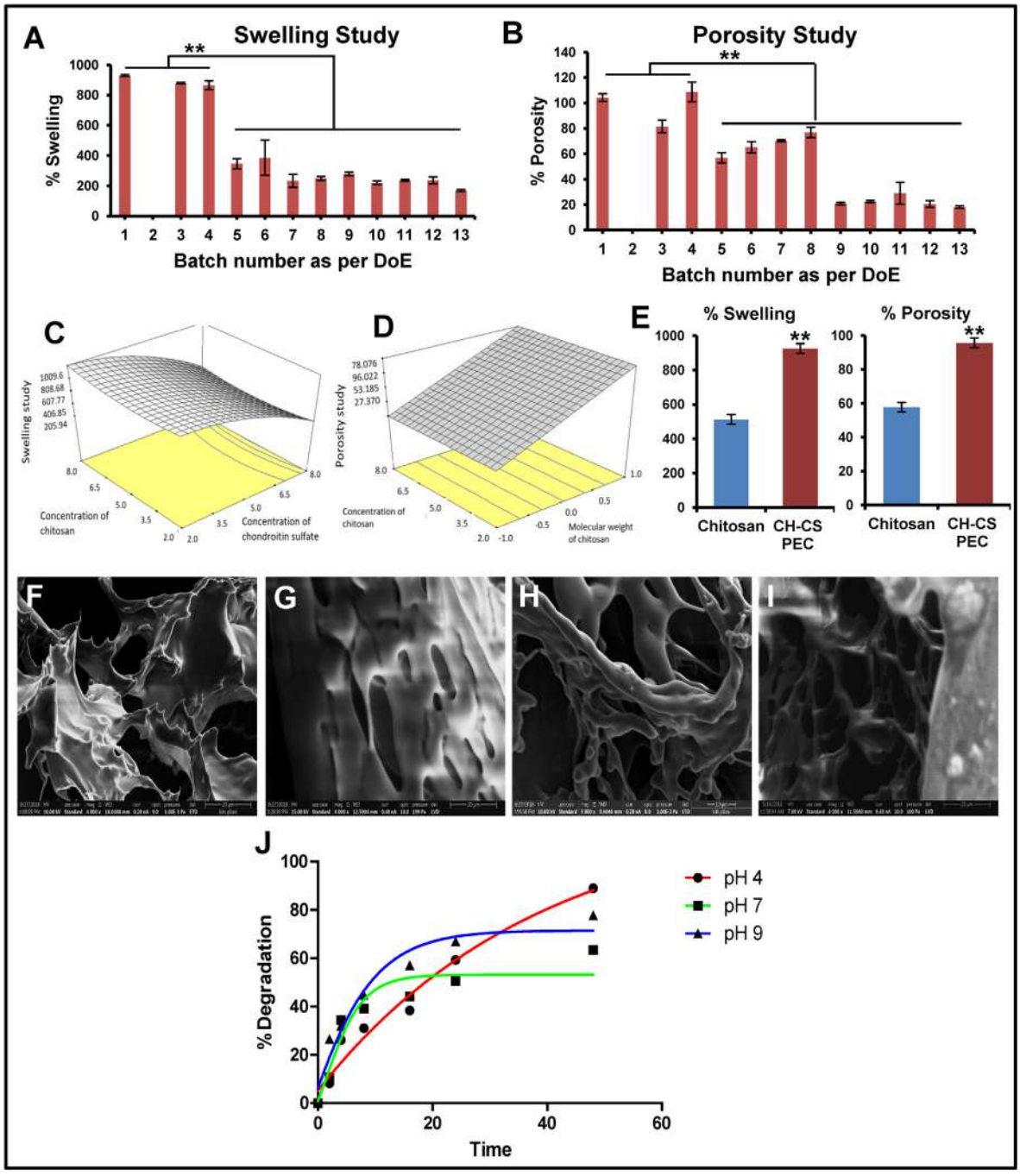

Fig. 3. Optimization of the CH-CS-PEC design based on swelling and porosity analysis. A. % Swelling and B. % Porosity of different batches. C. 3-D contour plot of concentrations of both the polymers vs. % swelling. D. 3-D contour plot of concentration of CH and molecular weight of CH vs. % porosity. E. Comparison between CH scaffold and the optimized CH-CS PEC in terms of swelling and porosity. F–I: SEM analysis of CH scaffold (F), and CH-CS PEC prepared with LMW CH (G), MMW CH (H) and HMW CH (I). J. Degradation kinetics of CH-CS PEC at different pH conditions. ** represents a significant difference ($p < 0.05$) as compared to other groups.

selected randomly among the combinations and were evaluated for the dependent variables. The performed dependent variables should match with the predicted solutions obtained by the software. The % prediction error should be <5% for the design to be validated. As depicted in Supplementary Table 1, the predicted error was found to be <5% in all the tested samples, validating the design analysis.

Based on the observed effect of the influence of MW of CH, and concentration of both CH and CS on the properties of the CH-CS-PEC, we have selected high MW CH and 5 wt% concentration for both CH and CS for our further study. For the physicochemical evaluation of the prepared CH-CS PEC scaffold, the prepared PEC was lyophilized for 24 h to make it dry and dried PEC was used for analysis.

3.6. Comparison of %swelling and %porosity of the CH-CS PEC scaffold with CH scaffold

A formulation chosen for wound dressing should provide adequate porosity for the gaseous exchange and to maintain a moist environment. CH is a natural polymer, which undergoes formation of hydrogel at acidic pH. Due to the gelling properties of CH, the polymer provides a suitable moist and porous environment for the healing of wounds. To determine the difference between the CH-CS PEC and CH scaffold, swelling and porosity of the optimized CH-CS PEC were compared with that of scaffold prepared with only CH (Fig. 3E). The prepared CH-CS PEC showed better swelling and porosity properties as compared with that

of the scaffold prepared with the same concentration of CH alone. Fig. 3E showed the % porosity and swelling of CH scaffold and CH-CS PEC. Only CH scaffold exhibited a swelling of ~510% and porosity of ~58% whereas CH-CS PEC exhibited a swelling of ~925% and porosity of ~95%. This could be because of the amorphous structure of the CH gel and lack of void volume in the gel. The cross-linking of CS with CH in the CH-CS PEC was probably lead to an increase in swelling and porosity of the CH-CS PEC than that of the scaffold prepared from the same concentration of CH alone.

3.7. Morphology of the CH-CS PEC

The morphology of the lyophilized PEC samples was studied using scanning electron microscopy (Fig. 3F–I). The images of the CH (high molecular weight, 5 wt% concentration), scaffold (Fig. 3F) and CH-CS PEC made up with 5 wt% concentration of low molecular weight (Fig. 3G), medium molecular weight (Fig. 3H) and high molecular weight CH (Fig. 3I) along with 5 wt% of CS was taken to check the cross-linked structure and pore size of the formed PEC. The images were taken at 20 μm scale at high vacuum mode in an Apreo-S FEI instrument. The images showed a change in pore size of the scaffold with different molecular weight of CH. The scaffold prepared with only CH was brittle in nature and the morphology showed uneven structures microscopically, with absence of distinct pore formation (Fig. 3F). In the CH-CS PEC, the structure was found to be much less brittle physically as well as more uniform in the microscopic analysis. With low molecular weight CH, however, the pores were less definite (Fig. 3G) compared to the PEC prepared with higher molecular weight CH (Fig. 3H–I). As indicated in the %swelling and %porosity studies, the pore size increased proportionately based on the molecular weight

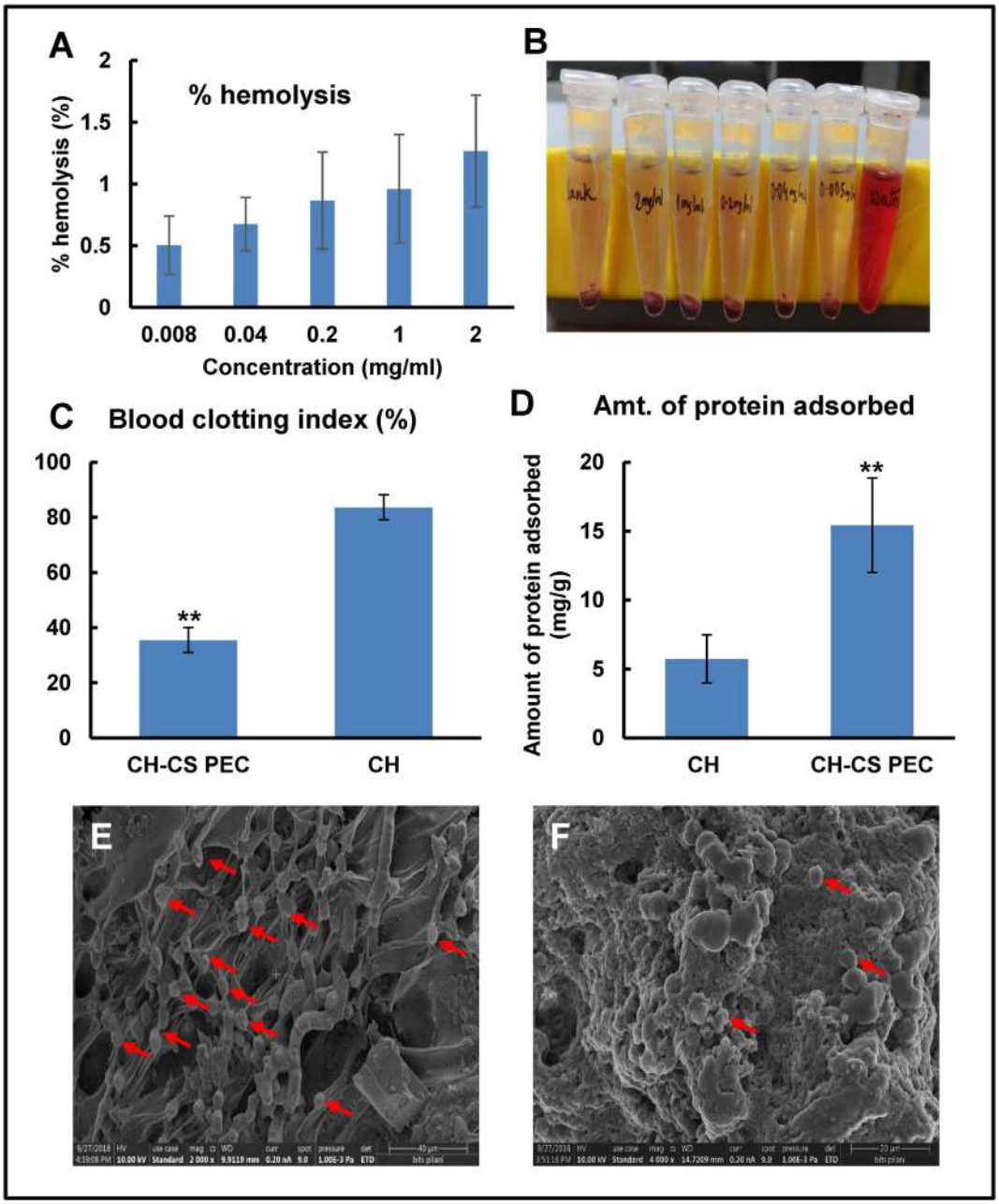

Fig. 4. Determination of hemocompatibility and hemostatic ability of CH-CS PEC. A. Hemolysis study. B. Pictorial representation of the hemolysis. C. Comparison of blood clotting index and D. protein adsorption on CH-CS PEC with that of CH scaffold. E. SEM image of blood cell adhesion on CH-CS PEC and F. that on the CH scaffold. Cells were indicated with arrow. ** represents a significant difference ($p < 0.05$) as compared to other groups.

of CH and high molecular weight CH exhibited the best porosity, which is also corroborated by the SEM analysis (Fig. 3I).

3.8. % degradation studies

Next, we wanted to study the stability of the CH-CS-PEC at different pH range. Stability was determined by quantifying the amount of amine groups released from the PEC in the aqueous medium. Quantification of CH-CS PEC was done using the 8% ninhydrin solution. Ninhydrin reacts with primary amino groups to produce a colored product, 2-(1,3-dioxoindan-2-yl) iminoindane-1,3-dione, also known as Ruhemann's purple. % degradation was calculated based on the formation of Ruhemann's purple complex from the amino group. As CH is the main scaffolding polymer present in the CH-CS-PEC, release of CH from the PEC would indicate breakdown of the composite structure of PEC. Degradation studies were performed at different pHs (pH 4, pH 7 and pH 9) up to 72 h. Higher rate of degradation was found with both high and low pH, with almost 90% degradation in 48 h at pH 4 and 80% at pH 9, whereas at neutral pH (pH 7), only about 50% degradation was detected in 72 h incubation (Fig. 3J). This data establishes the usability of the CH-CS-PEC at physiological pH. Faster degradation of the CH-CS-PEC at both acidic and alkaline pH may be due to the high rate of ionization of the scaffolding material (CH at acidic pH and CS at alkaline pH), resulting in breakdown of the composite structure.

3.9. In vitro hemolysis study

Biocompatibility is one of the most important properties of a wound-healing scaffold. As they directly come in contact with the blood, hematological stability is a good indicator of their biocompatibility. It is regarded that lower hemolysis is an indicator of better blood compatibility [36]. According to the American Society for Testing and Materials (ASTM), any material would be called non-hemolytic if it causes below 2% hemolysis [37]. We tested different concentrations of prepared CH-CS-PEC ranging from 2 mg/ml to 0.008 mg/ml. Maximum hemolysis was observed with 2 mg/ml CH-CS-PEC at 1.26 ± 0.45% (Fig. 4A–B). So the prepared CH-CS-PEC scaffold was considered as a non-hemolytic material.

3.10. Whole blood clotting and cell adhesion

Another very important criterion for a good wound dressing material is its hemostatic ability. It is particularly important for the treatment of open wounds which are bleeding. Induction of thrombosis can arrest the bleeding and stop blood loss. We evaluated the hemostatic potential of the CH-CS-PEC and compared it with CH scaffold on the basis of its blood clotting index (BCI). A smaller value of BCI indicates stronger hemostatic potential of the material. CH-CS-PEC demonstrated significantly higher hemostatic ability (35.46% BCI) compared to CH scaffold (83.64% BCI) (Fig. 4C). The higher hemostatic ability of the CH-CS-PEC compared to CH scaffold may be due to the higher porosity and swelling of the CH-CS-PEC compared to CH. Blood cell adhesion study was performed using scanning electron microscopy. The samples were treated in the same manner as that of treated for the blood clotting studies. Both CH-CS PEC and CH scaffold treated with blood were fixed with 4% formaldehyde solution for 15 min and dried at 37 °C. The SEM images of the blood pretreated scaffold of CH and CH-CS PEC showed that CH-CS PEC showed a better blood cell adhesion (Fig. 4E) in comparison to the scaffold prepared with CH alone (Fig. 4F).

3.11. Protein adsorption studies

After demonstrating high hemostatic activity with the CH-CS-PEC, we wanted to find the underlying cause for this. It is generally accepted that the first step of thrombosis formation is the protein adsorption on the surface of the scaffold. Better hydration and increased water absorption improve protein adsorption capacity of any scaffold. The aqueous layer is then replaced with the adsorbing protein molecules with the formation of a new 3D interphase. In this way, as the water absorption capacity of the scaffold increases, protein adsorption improves [38]. In agreement with the % swelling data, significantly higher amount of BSA was found to be adsorbed on the CH-CS PEC (15.4 mg/g) than that of CH scaffold (5.7 mg/g) (Fig. 4D), as the water uptake capacity of the CH-CS PEC is higher and may lead to better thrombogenic property. The protein adsorption studies showed that the CH-CS PEC exhibited almost three-fold increase in protein adsorption potential than that of scaffold prepared with CH alone.

3.12. Antibacterial study

As bacterial growth at the wound site is one of the most challenging factors for effective management of wound, inhibiting bacterial infection is a very important parameter for a wound healing material. Various studies have demonstrated that CH has antibacterial activity [39]. In order to determine the effect of PEC formation on the antibacterial activity of CH, we tested the antibacterial property of CH-CS PEC against

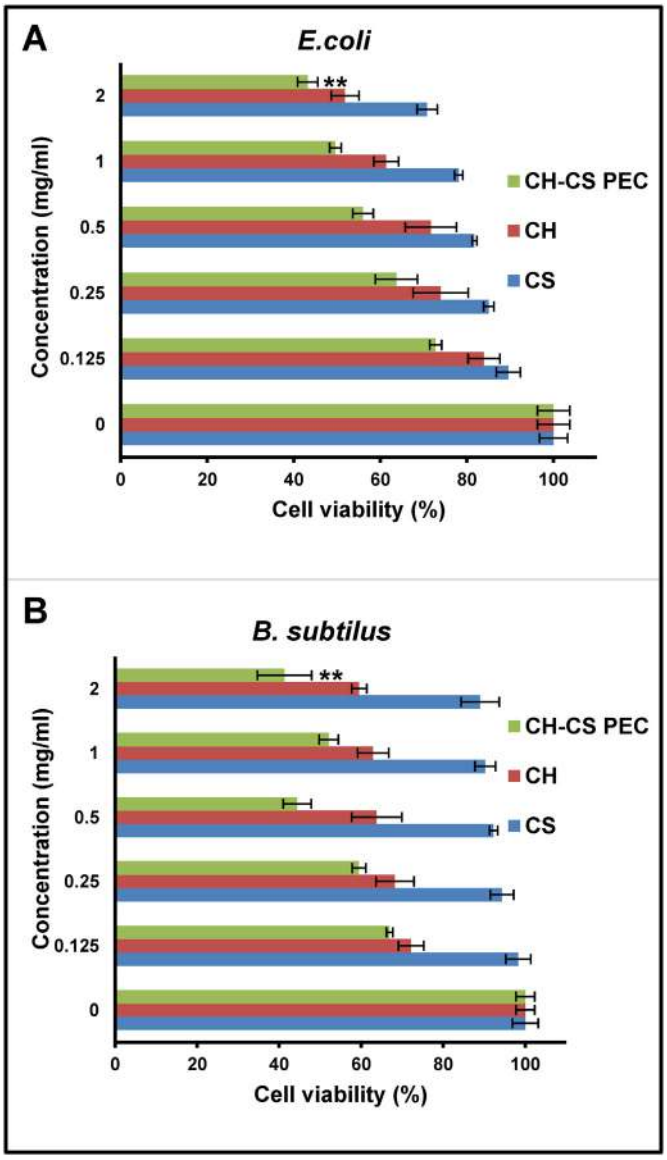

Fig. 5. Antibacterial property of the CH-CS PEC. A. % Cell viability against *E. coli* and B. against *B. subtilus*. ** represents a significant difference ($p < 0.05$) as compared to other groups.

one representative gram-positive and gram-negative bacteria, *Bacillus subtilus* and *Escherichia coli*, respectively, using the broth microdilution method. This method quantifies the number of viable bacterial cells on the basis of the intensity of formation of orange-red formazan crystals when the cells were treated with TTC dye. The concentration range studied was from 2 mg/ml to 0.125 mg/ml, as previously done with other CH based wound healing material [40]. As depicted in Fig. 5, both CH, CS and CH-CS PEC showed dose dependent antibacterial effect. Scaffold made with CH alone showed good antibacterial activity against both the gram-positive and gram-negative bacteria with about 50% viable cells against *E. coli* (Fig. 5A) and about 60% viable cells against *B. subtilus* (Fig. 5B) with 2 mg/ml dose compared to control. CH-CS PEC exhibited slightly better antibacterial activity compared to CH with about 45% viable cells against *E. coli* (Fig. 5A) and about 40% viable cells against *B. subtilus* (Fig. 5B) at 2 mg/ml dose. CS alone was found to have moderate antibacterial activity compared to both CH alone and CH-CS-PEC (70% viable cells against *E. coli* and 85% viable cells against *B. subtilus* at 2 mg/ml dose). Antibacterial activity of CS has been reported earlier [30,41]. The improved antibacterial activity with CH-CS PEC compared to CH alone may be due to an additive effect of both CH and CS. There are many different types of CH based wound dressings that have been evaluated previously. In many of these studies, active pharmaceutical ingredients have been incorporated in the scaffold for enhanced antibacterial activity [11]. However, we wanted to make a simple scaffold with the possibility of incorporating active drugs in the future. A better antibacterial activity of the basic scaffold would be highly beneficial. Many groups have chemically modified CH for the preparation of wound healing scaffold, however in many of these scaffolds, a loss of antibacterial activity has been observed compared to CH alone [42]. Alteration of CH structure has been argued to be the reason for the loss of antibacterial activity. To maintain the native structure and function of CH, we prepared the scaffold by only electrostatic

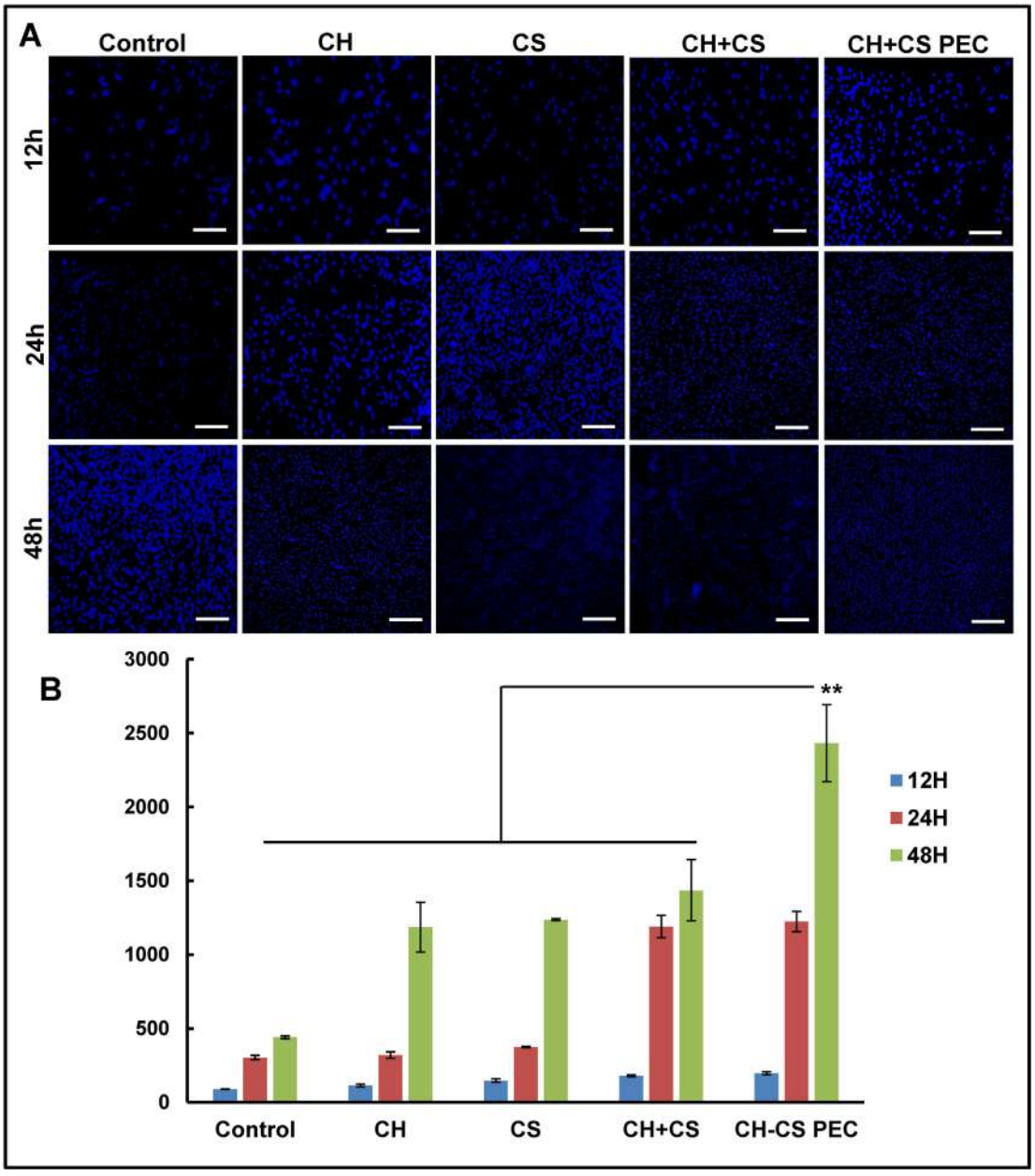

Fig. 6. Cell proliferation study with CH-CS PEC. A. Fluorescent microscopic image of the cells after specific time points. Scale bar, 100 μm. B. Comparison of cell number after specific time points. ** represents a significant difference ($p < 0.05$) as compared to other groups.

interaction, which did not adversely affect the bio-function of the polymer. Rather, mixing up of CH and CS in the CH-CS PEC exhibited an additive effect for antibacterial activity.

3.13. Cell proliferation studies

Next we wanted to check the cell proliferation with the treated CH-CS PEC in-vitro. Cells were first seeded on the scaffold coated (CH or CH-CS PEC) glass cover-slips. For treatment with CS alone, cells were seeded on the glass cover-slips and then it was treated with CS solution. As CS is of low molecular weight and does not make a scaffold on its own, we treated it like this. For CH scaffold + CS solution, cells were first seeded on the CH scaffold coated cover-slip which was then treated with CS solution. Control cells were grown on glass cover-slip only. The cell proliferation was observed at 12 h, 24 h and 48 h interval using a fluorescent microscope (Fig. 6A). Cell proliferation rate was calculated on the basis of the increase in number of cell count. The number of cells was calculated by quantifying the DAPI stained nucleus from the fluorescent microscopic image by ImageJ software. Cell proliferation was found to be highest with CH-CS PEC compared to other groups (Fig. 6B). At 48 h time point, the average cell number in the CH-CS PEC group was 2430, whereas with CH scaffold, CS solution and CH scaffold + CS solution, the average cell number was found to be 1185, 1238 and 1435, respectively. Significantly higher proliferation was observed with CH scaffold + CS solution compared to CH alone and CS alone till 24 h time point (with CH and CS alone, average cell number at 24 h time point was 319 and 374 respectively, whereas that with CH scaffold + CS solution was 1189), which, however, levels off at 48 h. Chitosan and chondroitin sulfate both are reported to have cell proliferation stimulating activity. Chitosan helps in myofibroblast differentiation and fibroblast proliferation [11,43]. Chondroitin sulfate is a glycosaminoglycan present in the extracellular matrix. It enhances cell proliferation by upregulation of FGF-2 and other cell-cell adhesion proteins [44–46]. As both the polymers have fibroblast proliferation potential, the combination probably has a synergistic effect. Between CH scaffold + CS solution and CH-CS PEC, they exhibited a similar level of cell proliferation till 24 h, may be due to the additive effect of the two polymers. However, at the 48 h time point, CH-CS PEC showed significantly higher cell density. This may be because of the better scaffold structure of the CH-CS PEC compared to the scaffold prepared with CH alone, as evident from the porosity study and SEM images. There are some reported findings in which combination of two polymers showed better cartilage regeneration [47]. However, the exact cellular mechanism behind this enhanced cell proliferation observed with the CH-CS PEC is not clear and beyond the scope of this article.

4. Conclusion

We have demonstrated that high molecular weight CH and CS solution can spontaneously mix to form a PEC. This property can be highly beneficial for developing an in-situ scaffold for wound healing purpose. We have established that the physicochemical properties of the PEC is greatly influenced by the molecular weight of CH and moderately affected by the concentration of both CH and CS. Higher molecular weight CH has exhibited better porosity and swelling. The optimum concentration for both CH and CS was found to be 5% w/v in terms of porosity and swelling characteristics. The CH-CS PEC was found to be hemocompatible with <2% hemolysis at the highest concentration studied (2 mg/ml). It also exhibited significantly higher blood clotting ability and protein absorption capacity compared to CH scaffold. The CH-CS PEC also showed a good antibacterial activity against both gram-positive and gram-negative bacteria. In vitro cytotoxicity studies in L929 fibroblasts cells revealed that the CH-CS PEC to be cytocompatible and can stimulate cell growth, which is highly beneficial for tissue regeneration and wound healing. These data indicate that the CH-CS PEC has high potential to be developed into an ideal wound dressing material.

Both the polymers used in the study showed non-over lapping beneficial effects for wound healing, and the combination leads to synergistic efficacy for effective chronic wound management. The available market products acts as either a bacterial barrier at the wound site, or it enhances tissue regeneration for effective wound healing; while the proposed formulation promises to function for simultaneous prevention of bacterial infection by acting as a biological barrier at the wound site as well as it promotes cell proliferation and tissue regeneration and helps to maintain homeostasis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.03.186.

Conflict of interest

The authors declare that there are no conflicts of interest.

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