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Microwave assisted chitosan-polyethylene glycol hydrogel membrane synthesis of curcumin for open incision wound healing

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Received November 2, 2019, accepted December 31, 2019

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Pharmazie 75: 118-123 (2020) *doi: 10.1691/ph.2020.9179*

Chitosan and polyethylene glycol hydrogel membranes containing curcumin were synthesized using microwave technology at fixed frequency, power and time of 2450 MHz, 500 Watt and 120 s. Polymers were solubilized separately, combined with drug and mixed in two different ratios i.e. F1=80:20 and F2=85:15. The untreated and microwave treated hydrogel membranes were analyzed for degree of swelling, degree of degradation, tensile strength, surface morphology, vibrational and thermal analysis and *in vitro* drug release. Results indicated that F2(micro) showed a significantly high degree of swelling (96.49±1.21 %), low degradation (9.88±1.68 %), sustained drug release through slow erosion (55.1±3.11 %) *via* non-Fickian diffusion. The vibrational and thermal analysis revealed rigidification of hydrophilic domains of the polymers by formation of hydrogen bonds between chitosan and PEG moieties (OH/NH) and elasticity of hydrophobic domains (asymmetric and symmetric CH moieties and/or C=O moieties) which not only significantly increased the transition temperature and enthalpy (297.2±3.2 °C and 4.24±1.4 J/g) of the chitosan moiety but also resulted in enhanced tensile strength (18.2±1.3 Mpa). *In vivo* wound healing study revealed significantly faster wound healing in the F2(micro) treated animal group in comparison to a control animal group where at day 14, a significant re-epithelization (87.26 %) with smaller wound size was observed. Hence microwave assisted chitosan-PEG hydrogel membrane of curcumin is advocated to be a suitable plate form for wound healing applications.

1. Introduction

The skin is the largest human organ which protects the internal human systems besides serving a barrier to the entry of exogenous substances. This barrier function can be compromised either partially or fully by different physical and/or chemical agents or due to accidents, abrasions etc. Wide research has been undertaken in the field of constructing skin tissue regeneration scaffolds to accelerate skin tissue regeneration. Hydrogels are best suited dosage forms for the purpose besides films, nanoemulsions, creams, gels which not only provide a moist environment may also be constructed to release medicaments in sustained fashion locally, thus providing a local antiseptic, antioxidant and anti-inflammatory effect. Commonly used hydrogels can also prevent infections and provide appropriate conditions for skin regeneration have some major disadvantages like poor mechanical strength, quick moisture loss or lack of biodegradability. Thus there is a demand for new, advanced biomaterials (Croisier and Jérôme 2013; Muxika et al. 2017; Muzzarelli et al. 2015).

Various natural drugs have long been used as dressings for wound healing namely honey, curcumin, *Aloe vera* and neem (Kateel et al. 2016). Curcumin is a yellowish compound; the main component found in turmeric which has a long tradition as a traditional spice in Asian countries with inherent antimicrobial, antioxidant, anti-inflammatory, anti-cancerous, anti-coagulant and hypoglycemic properties(Aggarwal and Harikumar 2010; Zhang et al. 2013). Curcumin has been found to promote tissue regeneration by tissue remodeling, granulation, new tissue formation and collagen deposition (Joe et al. 2004). Various carrier mediated drug delivery systems have been developed and tested for their potential in wound healing studies including polymer-curcumin nanoparticles (Chereddy et al. 2013; Krausz et al. 2015), polymer-curcumin nanoemulsion gel (Thomasa et al. 2017), gel (Kanta et al. 2014), self-assemble nanogel (El-Refaie et al. 2015) and hydrogels (Gong et al. 2013). Various attempts have also been made to develop such scaffolds in the form of sponges, polymeric films and nanofibers (Croisier and Jérôme 2013; Shi et al. 2016; Arockianathan et al. 2012). Curcumin has also been tested as a composite formulation for the purpose of accelerated wound healing applications (Cheng et al. 2015; Jayakumar et al. 2009). An ideal wound healing dressing must be able to absorb wound debris, reduce inflammation, prevent bacterial infection, reduce the loss of transepidermal water and aid in inducing cell proliferation to promote regeneration of the damaged tissue.

Chitosan, due to its poly(cationic) nature, as well as by the presence of hydroxyl and amino groups may create physical hydrogels with good sorption properties. Nevertheless, chitosan alone cannot address all the desired properties required for an ideal hydrogel wound healing preparation. Chitosan can undergo multiple chemical reactions resulting in the formation of branched structure *via* use of chemical cross linkers like glutaric aldehyde and genipin (Muzzarelli 2009; Piatkowski et al. 2018; Sudhanshu et al. 2017; Zazakowny et al. 2016). Chemical cross linking may enhance some properties of the hydrogels, which may nevertheless loose important properties like biodegradation and antibacterial activity (Croisier and Jérôme, 2013; Eweida and Marei 2015; Muxika et al. 2017). Furthermore, chemical cross linkers must be completely removed from the reaction mixture to prevent toxicity (Parhi 2017). Chitosan has a number of amino functional groups in its structure which can be activated in way to make them interact with other polymer functional groups to develop linkages (covalent and/or hydrogen bonds). However, a significant decrease in the number of NH_2 functional groups leads to a loss of antimicrobial properties of the chitosan (Ma et al. 2017). In view of these demerits, an alternative method of cross linking the polymers without the use of any toxic chemical agent and loss of desirable properties is needed.

Microwave represents electromagnetic waves having the frequency in the range of 300 MHz to 300 GHz (Surati et al. 2012). They tend to interact with the polar functional groups in volumetric manner (Abramovitch 2009). Microwave has been employed in the diagnosis and treatment of certain cancers (Rubæk et al. 2007), in endometrial ablation (Yeasmin et al. 2009), as transdermal permeation enhancer (Moghimi et al. 2010), as a technique to enhance local skin drug accumulation (Khan and Wong 2016), in tendons injury (Giombini et al. 2007) and as sterilization technique (Anuar et al. 2012). Owing to the fact that microwave can interact with polar moieties, it is thus investigated to be used for cross linking polymers to form a hydrogel structure.

2. Investigations, results and discussion

2.1. Swelling studies

Hydrogels are branched structures which can absorb high amounts of water and hence swell up which can be used to remove exudates from wounds. Figure 1 represents the swelling properties of untreated and microwave treated hydrogel membranes. It is evident that all hydrogel membranes showed excellent swelling properties especially those having higher quantity of chitosan in comparison to PEG 6000 (i.e. F2 and F2 (micro)). The significant increase ($p<0.05$, student's t-test) in the swelling behavior of the F2 formulation when it was treated with microwaves is explained by the fact that microwaves might have activated the polar functional groups of the chitosan (OH/NH, C=O) which led to interchain linkages between the chitosan and PEG and hence resulted in significantly enhanced hydrophilic character which led to the development of a sponge like structure able to absorb and retain high quantities of water (as shown in Fig. 2). The degree of deacetylation of chitosan is also directly proportional to its degree of swelling. Microwave irradiation may also have increased the degree of deacetylation in the polymer chain leading to high number of free -NH2 groups which are able to form hydrogen bonds among and/or with PEG chains.

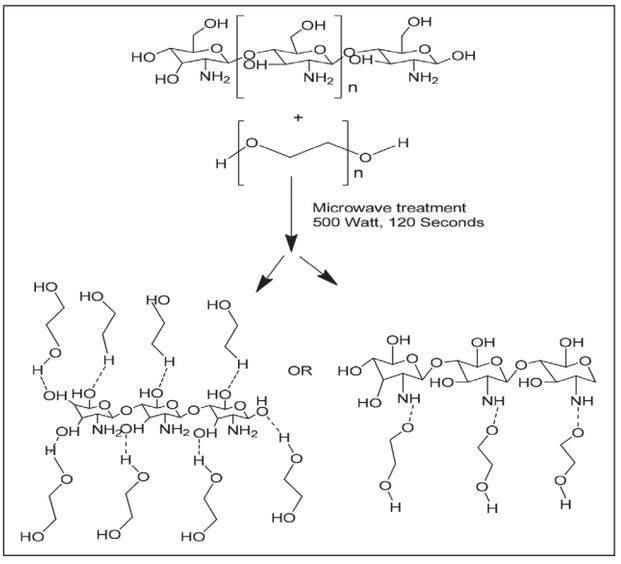

Fig. 2: Possible interaction mechanism of chitosan and polyethylene glycol following microwave treatment

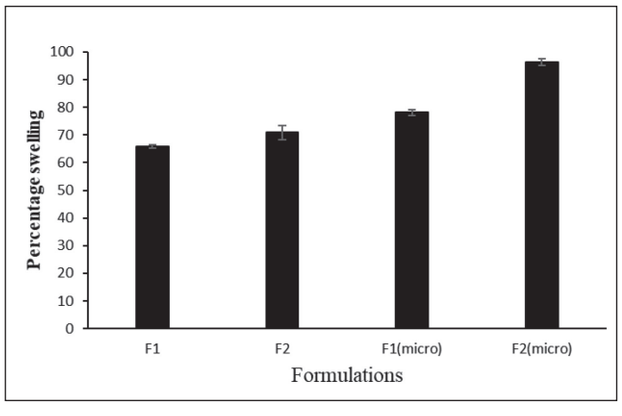

Fig. 1: Percent swelling of different hydrogel membrane formulations (n=3, ±SD)

2.2. Degradation study

The degradation behavior of the hydrogel membrane is necessary to investigate the possible fate when applied in *in vivo* models. Secondly, the hydrogel membrane must also be able to withstand the enzymatic attack when applied onto wounds and remaining there for a longer period. For this purpose, a degradation study of untreated and microwave treated hydrogel membranes was carried out in buffer pH 7.4 to mimic the open incision wound environment. The degradation results of untreated and microwave treated hydrogel membranes are shown in Fig. 3. The untreated hydrogel membrane formulations (F1 and F2) did not differ significantly ($p>0.05$, student's t-test) from each other despite of different concentrations of chitosan and/or PEG present. But when they were subjected to microwave treatment, degradation rate was significantly reduced ($p<0.05$, student's t-test) with more effect being observed for F2(micro) formulation. This could be due to the fact that the microwave elicited polar functional groups activation resulted in formation of much more compact structure leaving less or no voids in the polymer matrix hence delaying/preventing the penetration of more water molecules into the polymer matrix. Additionally, the high moisture content could be an additional reason which hindered the efficient breaking of the polymer into fragments as observed with untreated hydrogel membranes. Thus, higher swelling and less degradation behavior is deemed favorable for open incision wound healing applications.

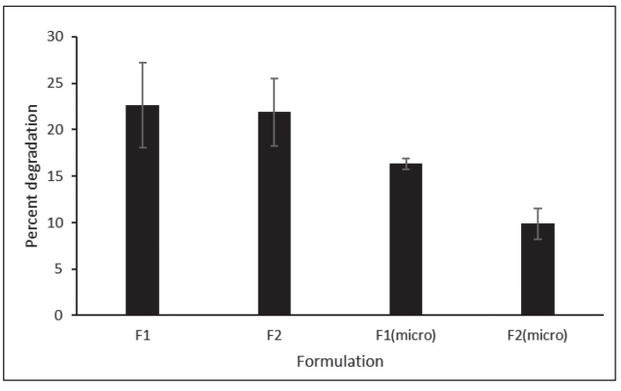

Fig. 3: Percent degradation of different hydrogel membrane formulations (n=3, ±SD)

2.3. Tensile strength

The tensile strength results are shown in Fig. 4. Characterization of mechanical properties is important because hydrogel membranes are required to be durable, stress resistant, soft, flexible, pliable and elastic in order to cope with the stresses exerted by different parts of the body (Boateng et al. 2008). The microwave treatment of the hydrogel membrane having chitosan-PEG in a ratio of 85:15 showed significantly higher tensile strength (student's t-test, $p<0.05$) than other formulations (F1) and untreated ones. The microwave treatment of F2 formulation increased the tensile strength to 18.2±1.3 Mpa compared to F1 which was found to be 15.3±1.2 while 10.12±1.1 and 11.2±1.3 for untreated F1 and F2. The enhanced mechanical properties of the F2(micro) can be explained by the fact that microwave treatment creates new intermolecular forces between the polar functional groups and increases chain entanglement density which in turn translates into increased flexibility (Kim et al. 2015; Zhai et al. 2003).

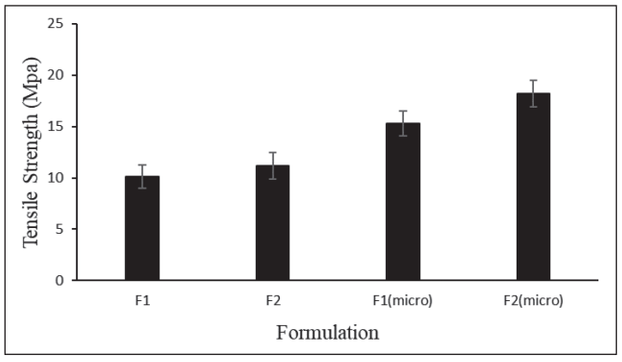

Fig. 4: Tensile strength of hydrogel membranes (n=3, ± SD)

2.4. Scanning electron microscopy

The surface morphological analysis of the optimized formulation (F2(micro)) in comparison to untreated F2 formulation is shown in Fig. 5. The results indicated that the untreated membrane formulation had widespread crystalline entities on the surface of the membrane which could consist of drug particles which failed to properly embed in the polymer matrix upon drying. They appeared to be individual entities lying on the surface, which could be the reason why the untreated membrane led to burst release of the drug during *in vitro* drug release studies. On the other hand, membrane synthesized with microwaves appeared to have a homogenous surface with drug properly embedded in the polymer matrix. Thus, microwave aided hydrogel membrane synthesis not only ensured homogenous mixing pattern of the polymers but also ensured successful embedment of the drug moieties in the matrix thus enabling drug release in sustained fashion for prolonged period.

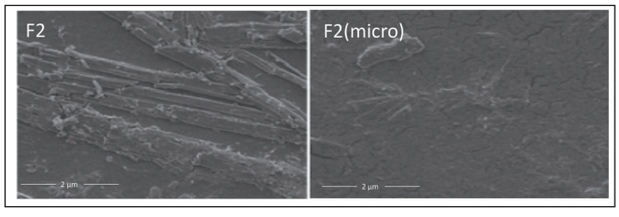

Fig. 5: Surface morphology of optimized untreated and microwave treated hydrogel membrane

2.5. Vibrational and thermal analysis

The vibrational analysis results of untreated and microwave treated hydrogel membranes are shown in Fig. 6. The results indicated that membranes formulated without microwave treatment resulted in the development of an amorphous structure with no significant difference in the corresponding wave number region of OH/NH (3090-3086 cm^{-1}) asymmetric (2921-2917 cm^{-1}) and symmetric C-H (2885-2865 cm^{-1}) and the amide-1 band (1546-1544 cm^{-1}) bands. The aliphatic C-H band (1427-1417 cm^{-1}) though experienced a significant increase (student's t-test, $p<0.05$) in the corresponding wave number in case of F2 formulation which might be due to the development of interactive forces between the chitosan and PEG at their aliphatic moieties due to the presence of higher PEG content in the formulation (Jayaramudu et al. 2016). Subjecting the same formulations (F1 and F2) to microwave treatment not only resulted in appearance of OH/NH wave number region which was not observed with untreated formulations but also resulted in shifting of C-H bands to higher wave numbers and merging of bands corresponding to asymmetric and symmetric C-H stretching for F2(micro) formulation. Microwaves are electromagnetic forces having the affinity to interact with polar moieties. Interaction with OH/NH moieties of the formulation resulted in a significant reduction in the wave number compared to F1(micro) indicating rigidification of the hydrophilic moieties due to development of new interactive forces between the chitosan polar functional moieties and PEG moieties, which is envisaged to contribute to an increase in the mechanical strength of the formulation (Fig. 4). Similarly, amalgamation of both polymers and deposition of polymer fibers in a specific pattern incited by microwave treatment resulted in shifting of symmetric C-H as well as aliphatic C-H moieties to higher wave number values (F2(micro), 2883.21±4.2)) indicates rigidification of hydrophobic domains of the polymer network by development of strong inter- and intra-polymer attractive forces between the chitosan and polyethylene glycol moieties. The summative rigidification effect is thus envisaged to translate into an increase in the mechanical strength and decrease in the degradation of the F2(micro) compared to F1(micro) (Fig. 4). Higher mechanical strength and slow degradation of the dressings are essential for efficient wound healing matrices.

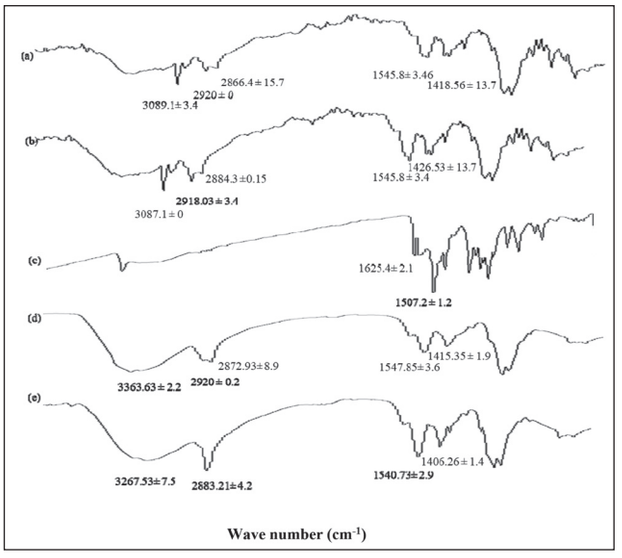

Fig. 6: ATR-FTIR spectra of a=F1, b=F2, c= curcumin, d=F1(micro) and e=F2(micro)

Thermal analysis of the formulations revealed a significant increase in the transition temperature as well as enthalpy of the chitosan moiety when treated with microwave for both formulations i.e. F1(micro) and F2(micro) (Fig. 7c-d) compared to untreated formulations (Fig. 7a-b). The microwave treatment of F2 formulation resulted in more rigidification of the polymer structure *via* development of new interactive forces between the chitosan and PEG moieties, where a significant increase in transition temperature as well as the energy required to induce transition of chitosan moiety up to 297.2±3.2 °C and 4.24±1.4 J/g was noted, while the transition temperature and enthalpy of the PEG moiety remains not much effected. Chitosan contains repeated units of 2-amino-2-deoxy-D-glucopyranose containing surface OH and NH_2 functional groups (Kittur et al. 2002) able to form additional linkages either hydrogen bonds and/or other interactive forces with the terminal OH functional groups of PEG, which is envisaged to increase the compactness of the resultant hydrogel membrane. The pure drug was also subjected to thermal analysis to elucidate the microwave effect on the drug, which remained unaffected. This is evident from the DSC thermogram with no significant changes induced in the transition temperature as well as enthalpy.

2.6. In vitro drug release

Appropriate drug release from the dosage form at the site of application is crucial to produce therapeutic response while sustaining the drug release over time ensures therapeutic effects over a longer period of time. The *in vitro* drug release behavior from the untreated and microwave treated hydrogel membrane formulations are shown in Fig. 8. The untreated hydrogel membranes (F1 and F2) released almost all embedded drug with in the first 6 h of the experiment with insignificant difference among both formulations ($p>0.05$, ANOVA)

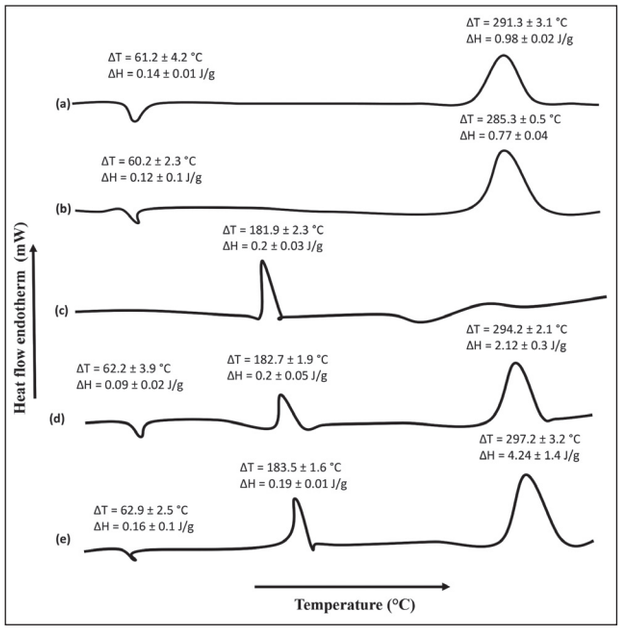

Fig. 7: DSC thermogram of a=F1, b=F2, c= curcumin, d=F1(micro) and e=F2(micro)

irrespective of the amount of either polymer in the formulation. The microwave assisted synthesized hydrogel membrane showed relatively sustained drug release pattern which were significantly different from each other as well as from untreated membranes ($p<0.05$, ANOVA). The F2 (micro) released almost 50% of the drug within 24 h time interval. Chitosan is a polycationic polymer which quenches hydrogen ions from acidic media thereby converting NH_2 to NH_3^+ thus enabling it to interact with the negatively charged moieties (PEG and/or drug). Curcumin has been found to actively interact with polar functional entities of the polymer molecules by readily forming bonds (Priyadarsini 2014). Thus electrostatic interaction between the drug and microwave activated polar functional groups of the polymer moieties in the hydrogel film is advocated to prevent rapid release of the drug from the matrix and hence sustaining the drug release in the dissolution medium.

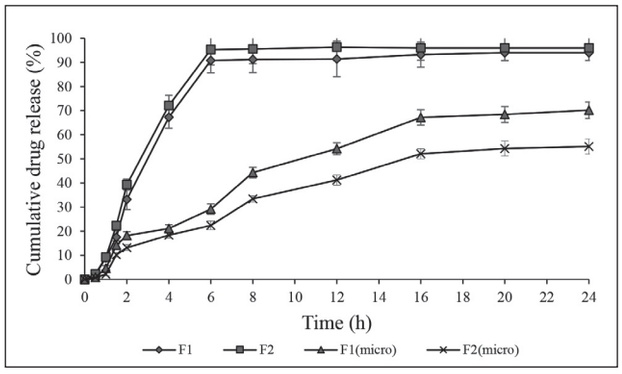

Fig. 8: *In vitro* cumulative drug release (n=3, ±SD)

When the drug release data were fitted into Korsmeyer Peppas equation, the value of n was found to be 0.420 for F1 and 0.398 for F2 which represents Fickian diffusion mechanism while the microwave treated formulations give rise to n values of 0.631 (F1(micro)) and 0.648 (F2(micro)) respectively representing Non-Fickian diffusion mechanism.

2.7. Wound healing study

Fig. 9a shows the rats with open incision wounds where the entire epidermis and dermis were surgically removed. The size of the wound gets significantly smaller with higher re-epithelization for the test group which was treated with curcumin hydrogel membrane (Fig. 9b and 9c, ANOVA: $p<0.05$). At day 0, the control and test group demonstrated complete skin barrier damage where both the epidermis and dermis are surgically removed, and internal tissue gets exposed to external environment with no protective skin barrier at place. During subsequent days, scab starts to develop in both groups covering the entire wound area and started to harden. At day 7, animals in both groups had fully developed scab but the F2(micro) hydrogel membrane demonstrated a remarkably fast reduction in wound size and higher re-epithelization propensity than the control animal group (ANOVA: $p<0.05$). The test group animals required 21 days to achieve complete re-epithelization while the control group needed 26 days.

Curcumin is a potent antioxidant and anti-inflammatory agent which has been found to promote skin tissue regeneration by tissue remodeling, granulation, new tissue formation and collagen deposition (Joe et al. 2004). Similarly, Chitosan has been found to promote cell proliferation, activate macrophages, stimulate tissue reorganization, promote fibroblast proliferation, promote collagen deposition, enhance increased production of hyaluronic acid at the wound site and also acts as hemostatic agent, thus helps in wound healing with minimal scar formation (Baldrick 2010; Paul and Sharma 2004). Thus, a synergistic effect of curcumin and chitosan translated into faster wound healing.

On the basis of above results and discussion, is is concluded that microwave treatment of chitosan-PEG matrix at a power of 500 W for 120 s not only enhanced the physicochemical attributes of the hydrogel membrane *via* establishing interactive forces between the two polymers through their polar moieties (OH/NH and/or C=O) but also promoted rapid wound closing and re-epithelization in animals. Keeping the wound moist by preventing excessive moisture loss, with sustained drug release, slower degradation and higher exudate adsorption is beneficial to promote skin tissue regeneration following damage. The optimized hydrogel membrane (F2(micro)) demonstrated to possess all the necessary properties to promote skin tissue regeneration.

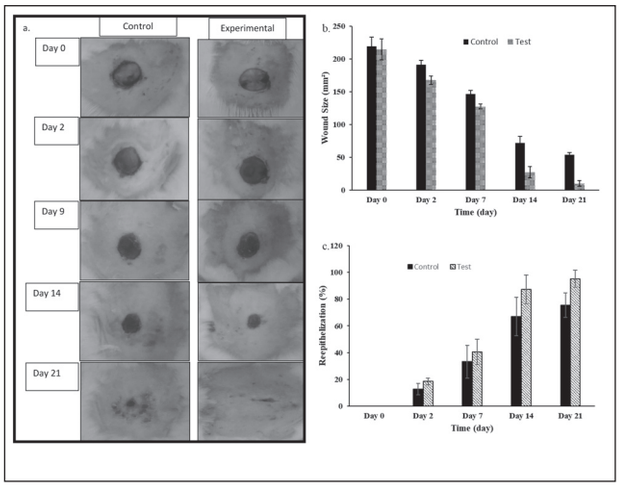

Fig. 9: a. Macroscopic wound images of rats with no applied scaffold (control) and treated by F2(micro) hydrogel membrane, b. Profiles of wound size and c. percent re-epithelization

3. Experimental

3.1. Materials

Chitosan (molecular weight ~ 310000-375000 Da, Sigma Aldrich, USA), polyethylene glycol 6000 (PEG, Sigma Aldrich, USA), curcumin (95% purity, Zhejiang Metals and Minerals, China), potassium dihydrogen phosphate (Sigma Aldrich, USA), sodium chloride (Sigma Aldrich), disodium hydrogen orthophosphate (Sigma Aldrich, USA). All chemicals were used without any further purification.

3.2. Preparation of chitosan/PEG hydrogel membranes

Chitosan and PEG hydrogel membranes were prepared by solution casting method. Briefly, 2 grams of chitosan were dissolved in 1 % (v/v) acetic acid solution to give

a 2 % w/w solution (solution A) and PEG 6000 was separately dissolved in distilled water at a concentration of 2 % w/w (solution B). A total of 1 g of curcumin was separately dissolved in 5 ml of 95 % ethanol. Both the polymer solutions were mixed in a specific ratio (F1 = 80:20 and F2 = 85:15) thoroughly and then the drug solution was added under continues magnetic stirring (Gallenkamp, England) until a homogenous mixture was obtained. The final 50 g of this mixture were added into a petri dish (internal diameter 88 mm) and subjected to microwave treatment in a household microwave (MS2022D LG, China) at fixed frequency of 2450 MHz at power of 500 W for 120 s and later placed in an oven (SH-DD-100NG, Korea) at 40 °C for 4 days and/or until complete drying. The untreated chitosan-PEG hydrogel membranes were developed using the same procedure. The untreated and treated microwave hydrogel membranes were then subjected to different physicochemical characterization tests listed below.

3.2. Swelling properties study

To determine the swelling properties of the obtained composite hydrogel membranes, both untreated and microwave treated hydrogel membrane samples were cut into specific dimensions (3 x 3 cm) and placed in a desiccator filled with anhydrous calcium sulphate for 24 h. Following complete drying, the dried samples were individually weighed (Wi). After that, the samples were transferred into a petri dish containing 20 ml distilled water and left again for 24 h to rehydrate completely. The rehydrated samples were weighed again (Wf) and the percent swelling was calculated from following relation;

$$\textit{Swelling degree}\ (\%) = \frac{Wf - Wi}{Wf} \times 100$$

At least triplicates were conducted, and results averaged.

3.3. Degradation study

The untreated and microwave treated hydrogel membranes were cut into square shaped strips (3 x 3 cm) and subjected to *in vitro* degradation study. For the purpose, weighed hydrogel membrane strips were immersed in 10 ml phosphate buffered saline pH 7.4 to mimic *in vivo* wound conditions and left for 24 h. The membranes were taken after 24 h, washed with water, dried and weighed again. The percentage of degradation was calculated using following relation;

$$D = \frac{w0 - w1}{w0} \times 100$$

where D = degradation degree, W0 = Initial weight of the analyzed sample, W1 = Sample weight after time t.

3.4. Tensile strength

The ultimate tensile strength (UTS) of the polymeric films was determined under ambient conditions using a universal testing machine Testometrics (United Kingdom). Three rectangular shaped strips with 7.5 cm length and 3.5 cm width were cut from each of the film samples and were fixed between the grips of the machine. The initial grip separation and cross-head speed were set to 50 mm and 5 mm/min, respectively. Sample was pulled with 50 N loads. The maximum force to break was recorded. Triplicates were conducted and the results averaged.

3.5. Scanning electron microscopy

The surface morphology of films was observed by field-emission scanning electron microscope (TESCAN Vega LMU, UK). A piece of 3 x 3 mm was cut from each film and adhered to stub with adhesive tape. The samples were then sputter coated with gold for 2 min followed by analysis on SEM at accelerating voltage of 10 KV. The respective sections were photographed at magnification of 20000x.

3.6. Vibrational and thermal analysis

The characteristic speaks of the dried polymeric films were recorded by an ATR-FTIR spectrophotometer (UATR TWO, Perkin Elmer, UK). Each film and/or powdered polymer was placed onto the surface of the diamond crystal and clamped to ensure close contact and high sensitivity. All the samples were scanned over a wave number range of 400 to 4000 cm^{-1} with an acquisition time of 2 min. Each sample was analyzed three times and results averaged.

The changes in the transition temperature of the polymeric film were recorded *via* differential scanning calorimetry (PerkinElmer Thermal Analysis, USA). An accurately weighed 4 to 6 mg of the polymeric film and/or powdered polymer was sealed in standard aluminum pan and heated from 0 to 300 °C under continuous flow of nitrogen gas at a rate of 40 ml/min. The characteristic peak temperature and enthalpy of the system were recorded. Each sample was analyzed three times and results averaged.

3.7. In vitro drug release

The *in vitro* drug release pattern of the curcumin from film samples was studied on Franz diffusion cell apparatus (Perme Gear, Inc. Model no: 4G-01-00-15-12). Briefly, the receiving compartment of the Franz diffusion cell internal volume (12 ml, surface area 1.76 cm^2) was filled with phosphate buffer saline (pH 7.4, to mimic open incision wound conditions) maintained at 32±2 °C with continued magnetic stirring at 400 rpm. The Tuffryn® membrane was used as a barrier between the receiving and donor compartments. The hydrogel membrane samples (1.76 cm^2) were placed on the membrane and the experiment was run for 24 h. Sample aliquots of 1 ml were withdrawn at regular time intervals of 0, 0.5, 1.5, 2, 4, 6, 8, 12, 16, 20 and 24 h and analyzed and replaced in equal volume at each sampling point to maintain sink conditions. Samples were analyzed on HPLC (Perkin Elmer system (UHPLC Shelton, CT, USA) consisting of an autosampler, Flexar FX-10, quaternary pump, degasser, Flexar FX UV/VIS UHPLC Detector, Chromera® software version 4.1.1.6396, C18 column (25cm × 4.6 mm i.d.; 5 µm; SUPELCO, Bellefonate, PA, USA). A reverse-phase HPLC assay was carried out using an isocratic system with a flow rate of 1.0 ml/min, a column temperature of 25 °C, a mobile phase of acetonitrile and 0.2 % acetic acid (80:20, v/v), and a detection wavelength of 425 nm. The injection volume was 10 µl. Solutions were filtered through a 0.45 µm nylon membrane (Merck Millipore, USA) prior to HPLC injection. The experiment was repeated three times for each hydrogel membrane formulation and results averaged.

3.8. Drug release kinetics

The mechanism of drug release from the films was assessed by fitting drug release data into Korsmeyer-Peppas equation as expressed by

$$Mt / M\infty = Kt^n$$

Where Mt / M∞ is a fraction of drug released at time t, k is the release rate constant and n is the release exponent. The 0.45 ≤ n corresponds to a Fickian diffusion mechanism, 0.45 < n < 0.89 to non-Fickian transport, n = 0.89 to Case II (relaxational) transport, and n > 0.89 to super case II transport (Dash et al. 2010).

3.10. Wound healing study

Healthy male Sprague dawley rats (weighing 250±5 g) were purchased from NIH Islamabad. They were acclimatized for 7 days with free access to food and water. All the animal procedures were approved from the institutional ethical review board (ERB#922/QEC/GU) adapting the international guidelines (OECD Environment, Health and Safety). The wounding of animals proceeded at day 8 by randomly dividing all animals into two groups (n-=6 each group) being first anesthetized with intramuscular injection of ketamine 90 mg/kg and xylazine 10 mg/kg body weight of rat, respectively. Subsequently, the hairs on the back of each animal were shaved with sharp blade and the shaved area was cleaned with an ethanol swab. Open incision wounds were created by marking the 15 mm area (wound diameter) on the shaved region followed by surgically removing the marked skin. Following wound infliction, the optimized hydrogel membrane was applied onto the wounded area with the aid of standard gauze and 3M adhesive tape. The control rats were defined as animals receiving only standard gauze application while test as animals receiving optimized hydrogel membrane (F2(micro)). The dressings were changed on a daily basis for 21 days. The surface morphology of the wound was recorded using a digital camera (Cannon D5100, Japan) at intervals of day 0, 2, 7, 14 and 21 in the absence of dressing and analyzed on image J to calculate the wound size. The percent re-epithelization was determined from the wound size profile using the following relation;

$$\textit{Percent reepithelization} = \frac{(\textit{wound size at time}\ 0) - (\textit{wound size at time}\ t)}{\textit{wound size at time}\ 0} \times 100$$

3.11. Statistical analysis

All experimental results are expressed as mean of at least three experiments with corresponding standard deviation. Statistical data analysis was carried out using SPSS software version 18.0 and a statistically significant difference was denoted by $p < 0.05$. Student's t-test and analysis of variance (ANOVA)/post hoc analysis by Tukey HSD test were employed where applicable.

Acknowledgments: The authors acknowledge the Fourth Military Medical University China, Ankang Central Hospital China, Gomal University DIKhan and Higher Education Commission of Pakistan for financial and facility support.

Conflict of interest: The authors declare no conflict of interest and all authors confirm agreement with the final statement.

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