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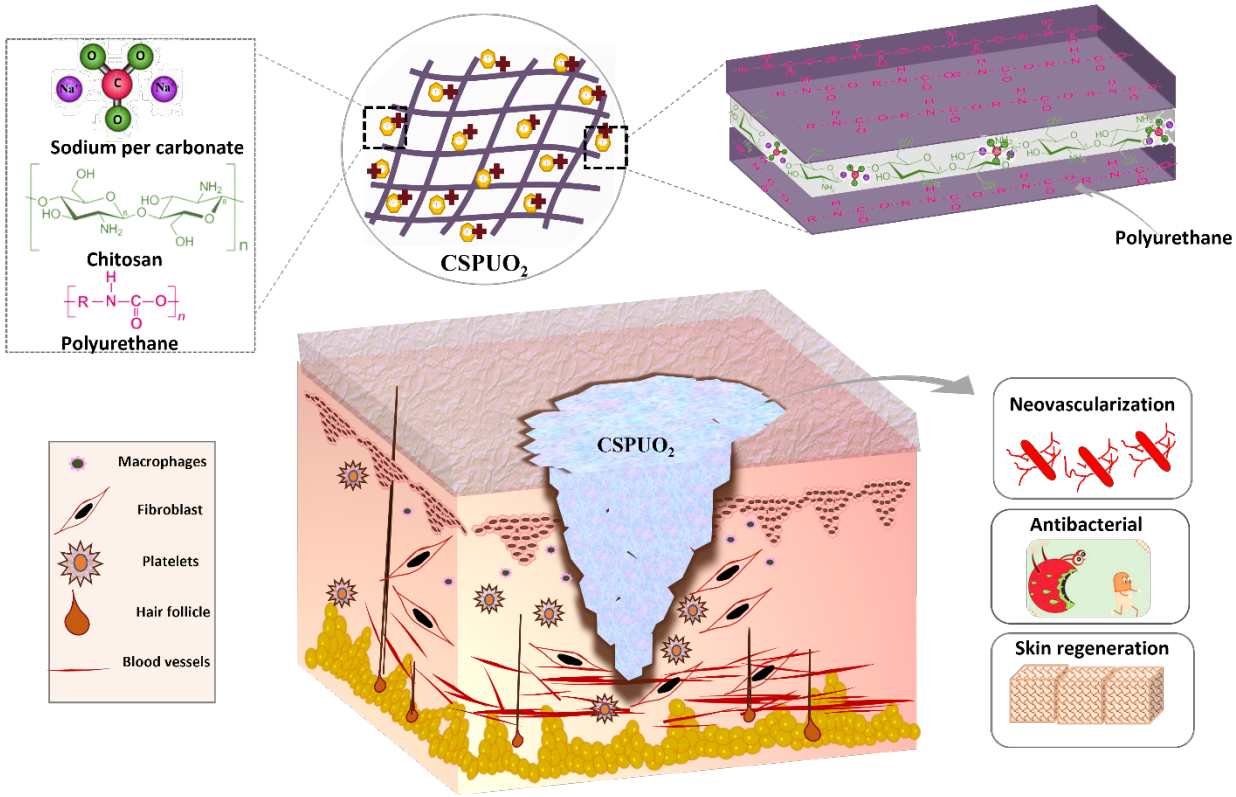
Graphical abstract



Chitosan-sodium percarbonate based hydrogels with sustained oxygen release potential stimulated angiogenesis and accelerated wound healing

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Abstract

The prolonged hypoxic conditions hinder chronic wounds from healing, and lead to severe conditions such as delayed re-epithelialization and enhanced risk of infection. Multifunctional wound dressings are highly required to address the challenges of chronic wounds. Herein, we report polyurethane coated sodium per carbonate-loaded chitosan hydrogel ($CSPUO_2$) as a multifunctional dressing. The hydrogels (control, CSPU and $CSPUO_2$) were prepared by freeze gelation method and the developed hydrogels showed high porosity, good absorption capacity and adequate biodegradability. The release of oxygen from the $CSPUO_2$ hydrogel was confirmed by increase in pH and a sustained oxygen release was observed over the period of 21 days, due to polyurethane (CSPU) coating. The $CSPUO_2$ hydrogel exhibited around 2-fold increased angiogenic potential in CAM assay as compared to Control and CSPU dressing. $CSPUO_2$ also showed good level of antibacterial efficacy against *E. coli* and *S. aureus*. In a full-thickness rat wound model, $CSPUO_2$ hydrogel considerably accelerated wound healing with exceptional re-epithelialization granulation tissue formation less inflammatory cells and improved skin architecture highlighting the tremendous therapeutic potential of this hydrogel as compared to control and CSPU to treat chronic diabetic and burn wounds.

Keywords: Oxygen releasing hydrogel, Wound healing, Sodium percarbonate (SPC), Angiogenesis, Hypoxia

1. Introduction

Chronic non-healing wounds are long-term complications as they significantly augment the risks of clinical infections, hemorrhage and amputation [1]. This poses an enormous burden to the healthcare system. In normal wounds, skin regeneration completes in four distinct but overlapping steps i.e., hemostasis, inflammation, proliferation, and remodeling. Healing of chronic wounds, however, infringes this chronology and halts in any phase owing to long-standing hyperglycemia [2]. Principally, an extended inflammation phase and delayed proliferation/angiogenic phase causes delayed wound healing. Hypoxia, too, is involved in delaying the wound healing. Firstly, it extends the inflammatory phase and elevates the wound infection risk by suppressing neutrophil-ROS mechanism. Secondly, hinders the aerobic cell respiration in hydroxylation of proline and lysine in the process of collagen synthesis. Finally, it blocks angiogenesis, epithelization and

wound closure [3]. The surge of deviant inflammatory responses disrupts the normal behavior of functional cells like endothelial cells, fibroblasts, keratinocytes, and even convicts injury and apoptosis to surrounding cells. Simultaneously, the recruited inflammatory cells at the wound bed generates bulk of reactive oxygen species (ROS), which intensifies the oxidative stress and consequently inaugurates numerous deleterious impacts, including halted collagen deposition and blood vessel regeneration, disordered degradation of growth factors and ECM, and delayed re-epithelialization [4]. Thus, it is critical to establish appropriate and effective approaches for the timely resolution of tumultuous inflammation, eradication of surplus ROS, and replacements of growth factors in the microenvironment of wound to accelerate the healing process [5]. As a way of dealing with this end, we designed an oxygen releasing hydrogel approach with the aim to avoid infection and promotes angiogenesis to ensure improved wound healing. Specifically, polyurethane stabilized sodium percarbonate based chitosan hydrogel with a sustained oxygen release was developed to locally deliver oxygen at wound area. This optimized oxygen release approach can ensure 1) skin regeneration and blood-vessel proliferation for improved wound healing 2) antibacterial abilities to inhibit wound infection. This approach is particularly designed to address the complex needs for regenerating the lesioned skin issues.

Controlled oxygen delivery to wounded site effectively hinders the generation of proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Oxygen has a remarkable potential to scavenge ROS and greatly augments the performance of antioxidant enzymes. However, the concentration availability and therapeutic feasibility of oxygen is still a challenge as high oxygen concentration may induce hyperoxia causing mitochondrial compartment destabilization, oxidoreductive processes and cell apoptosis. On the other hand, it plays a significant role in skin regeneration by enhancing cell proliferation and migration, and appropriate granulation of tissue and ECM [6]. However, uncontrolled topical administration of oxygen causes rapid delocalization in moist wound environment. In current study, to resolve this issue, oxygen generating specie (sodium percarbonate) was loaded on to chitosan hydrogel and coated with polyurethane to maintain the sustained release and appropriate concentration of oxygen at the wound area. Although the application of oxygen is reported to promote angiogenesis and ECM formation, its controlled/sustained delivery has not been studied in detail. Recently, few reports have been published on the precise and controlled release of oxygen and the impact of its controlled release on the wound healing [7].

In recent years, hydrogels have gained tremendous attention as wound healing dressings. Benefiting from inter-linked three-dimensional networks, hydrogels present a hydrated and occlusive microenvironment to enhance the penetration of nutrients and oxygen that is essential for angiogenesis and tissue regeneration. A variety of chemicals, proteins, small molecules, and living cells, can be easily loaded into the hydrogel network, completely protected from degradation, and released in a controlled pattern. As a natural polysaccharide having excellent gelation properties even without a gelling agent, biocompatibility, biodegradability and hydration ability, chitosan has been reported for angiogenesis promotion and skin regeneration, which offers an extraordinary potential as a wound healing material. Moreover, chitosan is associated with the management and treatment of chronic wounds owing to its non-antigenic, tissue-adhesive, antimicrobial and hemostatic characteristics, which subsequently led to its application as a commercial wound healing dressing [8, 9]

In this study, the simplest form of chitosan hydrogel was formulated and a physical loading method was used to load sodium per carbonate as the oxygen generating specie and was finally coated with polyurethane. In vitro experiments showed the extraordinary cell viability and cell migration ability in MTT assay and cell migration assay respectively. Moreover, the angiogenic ability was studied by CAM assay. The gradual and steady release of oxygen over the period of 21 days was detected through the oxygen detection assay that ensured the in vivo wound healing in animal studies. Oxygen was steadily released while the sodium per carbonate loaded hydrogel was applied to wound to timely initiate the angiogenic and proliferation phase and avoid the risk of getting infection. Thus, the released oxygen produced a significant therapeutic impact on a full-thickness wound model of rat. In short, the present study presents an easy to manufacture and affordable strategy for the sustained release of oxygen using a safe and hydrating hydrogel with angiogenic ability. We propose that the polyurethane coated sodium percarbonate based chitosan hydrogel ensures the regulated oxygen release to provide the suitable local concentrations of oxygen that greatly enhanced the antibacterial and angiogenic potential of hydrogels to treat chronic wounds.

2. Materials and Methods

2.1. Materials

Chitosan powder (CAS No. 9012-76-4) was purchased from Bio Basic (Canada). Polyurethane and tetrahydrofuran were purchased from Merck (USA). Sodium per carbonate was bought from Daejung Chemicals and Metals Co Ltd (South Korea). Phosphate Buffered Saline (PBS) in the

form of tablets of pH 7.4 was purchased from Bio World (USA). Nutrient broth and acetic acid were purchased from Sigma Aldrich (USA).

2.2. Preparation of polyurethane coated sodium per carbonate loaded chitosan hydrogels

A chitosan solution (3% w/v) was prepared in 1 % aqueous acetic acid. Completely dissolved solution was first frozen at −4 C, for 48 hrs. and then immersed in 3M solution of sodium hydroxide (NaOH) prepared in ethanol. After that, samples were refrozen at −4 C for another 48 hrs. Finally, washing was done with (75%, 50%, and 25%) ethanol solution, and finally washed with distilled water to achieve the pH = 7. The neutral samples were dried at room temperature.

Sodium per carbonate (SPC) an oxygen generating biomaterial was loaded on dried chitosan hydrogel. A 2% sodium per carbonate was loaded on the hydrogel. A 20 ml of 10% polyurethane solution was prepared in a 1:1 solution of tetrahydrofuran (THF) and dimethylformamide (DMF). Then it was divided into two equal parts (10ml each). One portion was used to prepare 2% sodium per carbonate (SPC) solution while the other was kept unchanged (simple polyurethane solution). For 2% solution 0.2g SPC was dissolved in the 10ml of polyurethane solution. Dried chitosan hydrogel was immersed in both solutions separately. Only polyurethane coated hydrogels were prepared to compare their effects having sodium per carbonate along with polyurethane. Table 1 shows the codes and final concentrations of all prepared hydrogels.

Table 1: Codes of prepared chitosan hydrogels with all their ingredients and their concentrations

No.	Code of hydrogel	Hydrogel ingredients and concentrations
1	Control	Chitosan hydrogel (3% chitosan solution in 1% acetic acid)
2	CSPU	Chitosan hydrogel with polyurethane coating (10% polyurethane solution)
3	$CSPUO_2$	Chitosan hydrogel loaded with sodium per carbonate and polyurethane coating (2% SPC in 10% polyurethane solution)

2.3. Morphological characterization

The morphology of the hydrogels was studied by a scanning electron microscope performed at Lahore University of Management Sciences (LUMS) Lahore. The sputter coated samples were placed on SEM holders at an accelerating voltage of 10 kV for imaging. Image J software was used for image-processing and the average parameter of 70 pores was measured from the obtained

SEM images. The deeper visible pores on the surface were also considered in the pore's parameter measurement. The average pore size was taken as an average of both the major and minor (the primary and secondary) axis of the best fitting ellipse to the pores.

2.4. Absorption studies

The hydrogels (n = 3) were weighed (M_d) and immersed in PBS solution (pH = 7.2) at 37 ˚C. At different time points, the hydrogel samples were taken out and excess water was removed from the surface of hydrogels with a filter paper. The degree of swelling was then measured by using the following formula:

$$\text{Degree of swelling (\%)} = [(M - Md) / Md] \times 100$$

Where M is the weight of each sample after submersion in the buffer solution and Md is the weight of the sample in a dry state.

2.5. Degradation analysis

Hydrogels (n = 3) were weighed (W1) and immersed in PBS, lysozyme (1 mg/mL) and H_2O_2 (3.5 M) solution at 37 ˚C. At different time points (day 7, 14, 28, 35, 42), the hydrogel samples were taken out dried and subsequently weighed (W2). The dried weight (without water content) remaining ratios were determined by the following formula:

$$\text{Dry weight remaining ratio} = W2/W1 - 100$$

2.6. Detection of oxygen generating ability of the hydrogel

Oxygen generation by biomaterial loaded on hydrogel was assessed by the change in the pH of the solution. For this, all three hydrogels (n=3) were immersed in PBS solution (pH=7) at 37 ˚C. The 4th sample SPC hydrogel (loaded with sodium per carbonate but not coated with polyurethane) was also immersed in PBS solution just to compare the oxygen release pattern from the coated and uncoated hydrogel. After every 24 h pH of the PBS solution was monitored by a pH meter for 21 days.

2.7. Cytocompatibility studies

2.7.1. MTT assay

In vitro, experiments on NIH 3T3 mouse fibroblast L929 cells were carried out by culturing cells with the gamma sterilized hydrogels. Cell line was maintained in Dulbecco's Modified Eagle Medium high glucose (DMEM-HG) supplemented with 15% fetal bovine serum (FBS). The medium was replaced with the fresh medium every three days. Briefly, cells were seeded (12×10^3 cells/well) into 24 well tissue culture plates. Gamma sterilized samples were submerged in the cell growth medium in the 24 well plates with the caution that the dressings were not in direct contact with the cells. After three and seven-days measurement was taken using an MTT assay kit (Biobase, USA). A 10µl of MTT solution (5mg/ml) was added to each well and incubated in CO_2 incubator at 37°C for 4 h. Then, the supernatant was discarded, and formazan crystals were dissolved with dimethyl sulfoxide (DMSO). A 100µl DMSO was added to each well and cells were incubated at 37°C in the cell culture incubator for next 2h. The absorbance was measured through a microplate reader at 570 nm wavelength. DMSO was used as a reference.

2.7.2. Wound scratch assay

NIH 3T3 cells were seeded (5×10^3 cells per well) in 6-well cell culture plates in DMEM-HG medium supplemented with 15% FBS for 24 h. A sterile 1000 µL pipette tip was used to scratch the confluent cell monolayer. The cells were incubated with SPC supplemented serum free cell growth medium at 37°C in the cell culture incubator. Scratch wound models were observed under a phase-contrast microscope after 4 and 24 hours and images were taken to analyze the cell migration in each group.

2.8. Analysis of antibacterial potential by turbidimetric method

S. aureus and *E. coli* were used to analyze the antibacterial effects of all three chitosan hydrogels. The sterile broth media was placed in sterile test tubes and the inoculation of both bacterial strains was added separately to each test tube. After 24 h of incubation time hydrogels (Control, CSPU, and $CSPUO_2$) of 2×2 cm^2 size immersed in the media. Absorption was taken after 4, 8, 24, 48 and 72 h at 600nm through a spectrophotometer.

2.9. Angiogenic potential analysis by *in ovo* Chorioallantoic membrane (CAM) assay

Fertilized chicken eggs of 6^{th} day age were purchased from Big Bird Hatchery (Lahore, Pakistan) incubated in the egg incubator (R-COM Suro20) at 37.8 °C and 55% humidity. The next day (7^{th}

day of fertilization), a square window of 1 cm^2 was made into the shells of all eggs. All pre-gamma sterilized samples were implanted separately into the CAM of each egg (n=10). After implantation, the shell windows were sealed with parafilm (Bemis Flexible Packaging, USA) and adhesive tape. Eggs were again incubated under the same conditions. On day 14, eggs were opened and evaluated. Scaffolds were retrieved. Images were taken with a high-resolution digital camera. Blood vessels in each CAM were quantified and processed through the Image J software. The results of 7 chicks, that survived out of 10 fertilized eggs from each group were presented as mean ± S.D. The retrieved implants were preserved in 4% paraformaldehyde solution at 4°C temperature for histological assessment. The implants retrieved from the CAM assay were processed using 4 ethanol gradients (100%, 90%, 70%, and 50%) and xylene. Processed samples were then embedded in wax and tissues were sectioned (5µm) by using a microtome. Tissue sections were stained with Hematoxylin and Eosin stains. Stained sections were observed under a phase contrast microscope and images were taken.

2.10. *In-vivo* wound healing potential

For experimental procedures of *in vivo* wound healing analysis approval was obtained from the ethical Committee of Animal Care, National Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. Fifteen female Wistar rats, aged 10-12 weeks and weight 150 gram were randomly divided into three groups (n=5) Control group, CSPU treatment group and $CSPUO_2$ treatment group. All animals were kept in an animal care facility under controlled climate conditions (humidity 0% to 70% and temperature 22°C) with a 12 h light/dark cycle, free access to filter-sterilized water, and standard rodent laboratory feed.

The rats were anaesthetized before the surgical procedure to create wounds with ketamine (90mg/Kg)/xylazine (15mg/Kg) anesthesia. Anesthesia was administrated intramuscularly according to rat body weight (0.81/g). A sterilized circular template (2 cm x 2 cm) was placed on the shaved backs of the rat and a full-thickness excisional wound extended through the subcutaneous tissue, including the panniculus carnosus was created by using serrated forceps and iris scissors. Designated hydrogel per group was applied over the wounds and secured with the help of surgical tape. Primary dressing (hydrogel) was maintained from day 0 to day 5, then it was taken off and replaced after every 5th day for imaging (10, 15, 20, 25, and 29). On the planned days (days 0, 5, 10, 15, 20, 25, 29) all the experimental rats were anaesthetized and firmly positioned on a dissecting board with the wound facing upwards. The margins of the wounds

(edges of wound closure) were traced onto a glass microscope slide with a fine tip permanent marker and photomicrographs were taken at a fixed distance and angle from the wounds. Wound sizes were measured at each designated above-mentioned time point from the traced margin of the wounds taken immediately after wounding and reported as a percentage of wound closure as calculated by using the following formula

The % of wound closure = wound area on day 0 - open wound area/wound area on day 0 × 100

On day 29, final images were taken, and all experimental rats were sacrificed.

2.11. Histological Evaluations

On the final day of animal studies, the healed skin tissues were collected from each rat for histopathological evaluation. The extracted samples were washed with PBS and preserved in 10% neutral buffer formalin. Then tissues were embedded into paraffin and sectioned into slides of 5-µm thickness by a microtome (HM-340E, Microm Inc. USA). Hematoxylin and Eosin (H&E) stained tissues were observed under a light microscope to assess the wound healing capacity of applied hydrogel.

2.12. Statistical analysis

Results of all experiments including swelling studies, degradation studies, MTT, wound scratch and CAM assay were statistically analyzed by one-way analysis of variance (ANOVA) and student t-test. Data were presented as mean and ± standard deviation. Individual two group comparison was examined with a student's *t*-test. A "P" value < 0.05 was taken as statistically significant.

3. Results

3.1. Preparation of hydrogels

In view of the dynamic challenges of chronic wounds, a chitosan-based hydrogel was prepared. Chitosan has inherent property to undergo the cross-linking reaction with another polymeric chain of its own that may be similar or different from the first chain. The amino and hydroxyl groups interact through hydrogen bonding and form the hydrogel structure [10, 11] (figure 1A). The simple self-cross-linked (through H bonding) chitosan not only ensured good physical properties but also reinforced biological efficacy of the designed hydrogels. Cross-linking and gelation rate

of chitosan enhance its adhesion and antibacterial ability [10]. Both wet and dry forms of chitosan hydrogel were soft and flexible (figure 1C&D). The dried hydrogel was loaded with sodium per carbonate and coated with polyurethane to prevent instant release of oxygen produced by sodium per carbonate. The overall hydrogel preparation, loading and coating is shown in figure 1B. The loading of sodium per carbonate and coating of polyurethane did not affect the flexibility or softness of hydrogel.

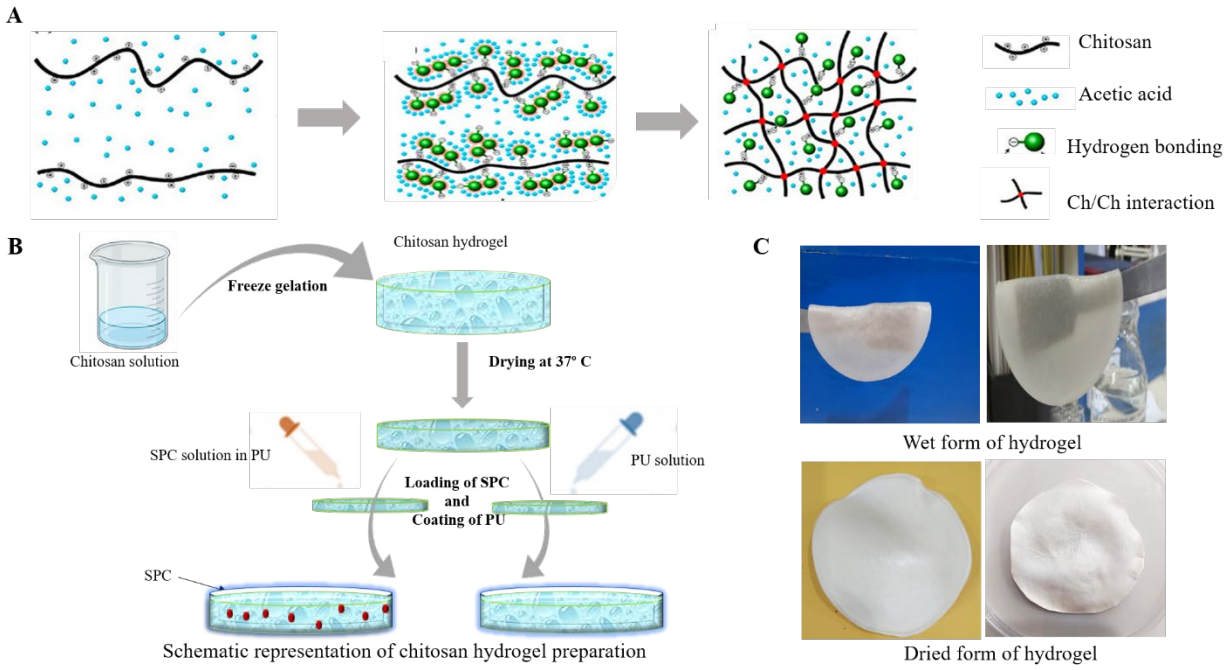

Figure 1. A. schematic representation of self-cross-linking of chitosan to form hydrogel. B. Schematic representation of overall scheme of preparation of polyurethane stabilized sodium per carbonate-based chitosan hydrogel. C. Wet form of chitosan hydrogel. D. Dry form of chitosan hydrogel.

3.2. Morphological analysis

A well-defined porous structure of hydrogel is quite essential for hydrogel to apply to wounds, as it ensures the easy access to water vapor and allows free transportation of oxygen and other necessary nutrients. It also influences cell infiltration, proliferation, and function in tissue engineering. The surface morphology of all three hydrogels revealed a porous structure (Figure 2A). The interconnected porous structure of the hydrogel was retained after sodium per carbonate loading and polyurethane coating. The mean pore size, of the Control hydrogel, was, 4.85± 2.26

μm. While the CSPU hydrogel showed a little decline in pore size as the average pore size was 3.73 ±1.92 μm. Further reduction in pore size was observed in the case of $CSPUO_2$ hydrogel as the mean pore size, in this case, was 3.35 ± 1.05 μm. It was also observed that there was a decline in the fibrous extensions in between pores and more sheet-like structures appeared post sodium per carbonate loading.

3.3. Absorption studies

The ability to absorb heavy wound exudates and retain the breathability is critical in designing the hydrogel dressing. Low absorption ability may cause leakage (known as strike-through) and increases the time of healing as well as the risk of infection as moist or wet surface of dressing is more attractive to microbes. The solution absorption capacity of control, CSPU and $CSPUO_2$ was performed in PBS solution. All hydrogels depicted time dependent swelling profile, which reached the maximum value after 4 days (figure 2C). After that, no more significant absorption was noted. It was observed that Control hydrogel exhibited 850% absorption capacity. While CSPU and $CSPUO_2$ possess lower absorption capacity (669% and 621% respectively) as compared to Control that is due to hydrophobic nature of polyurethane.

3.4. Degradation studies

Degradation assessment of biomaterials is important as it helps to examine the life of biomaterials inside the human body. In current study, we mimicked the physiological environment, by using lysozyme (1 mg/mL of PBS) and H_2O_2 (3.5 M H_2O_2 solution in PBS) media. Hydrogen peroxide is naturally present inside the human body and the amount of hydrogen peroxide plays a significant role in determining the sustainability of biomaterial. The data indicated that hydrogels possess greater degradation in degrading enzyme lysozyme and H_2O_2 solutions (figure 2 B). After 42 days in PBS solution, the Control sample's dry weight ratio was decreased to 15% (from 100% to 85%), in CSPU sample, decrease ratio was 12% (from 100% to 88%) and in $CSPUO_2$ decrease ratio was 13% (from 100% to 87%). In PBS/H_2O_2 solution Control showed decrease ratio of 17% (from 100% to 83%), CSPU showed decrease ratio of 16% (from 100% to 84%) and $CSPUO_2$ showed decrease ratio of 17% (from 100% to 83%). In PBS/lysozyme solution Control had a decrease ratio of 19%, both CSPU showed and $CSPUO_2$ showed 18% reduction ratio (from 100% to 82%).

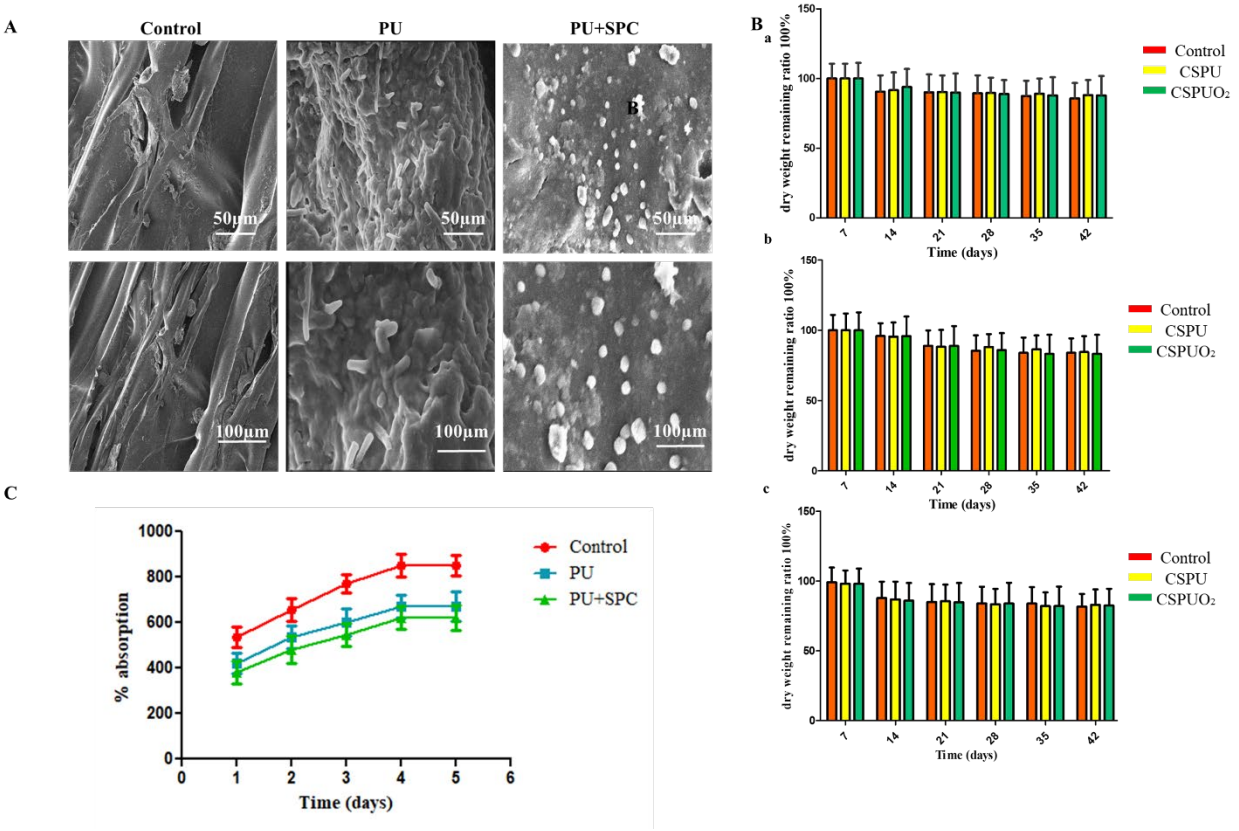

Figure 2. A. Scanning electron microscopic images of cross-linked hydrogels (magnification bars are given with each image). B. Measurement of the rate of degradation of hydrogels (Control, CSPU and $CSPUO_2$) in PBS (a), PBS+H_2O_2 (b) PBS+Lysozyme (c). C. Assessment of absorption capacity of all three hydrogels in PBS solution. Graphical data presented as mean (n=3) ± standard deviation of three independent experiments. C.

3.5. Detection of oxygen generating ability of the hydrogel

Detection of oxygen generating ability of oxygen releasing hydrogel was done by measuring pH of the solution in which hydrogel loaded with sodium per carbonate was immersed. Oxygen release in a solution increases its pH as oxygen produces more hydroxyl ions by attacking on hydrogen of water molecules through its lone pairs [12, 13] (figure 3C). Results (figure 3 A) showed that no change in pH was observed in case of Control and CSPU hydrogel as there was no oxygen generating agent such as sodium per carbonate. But the $CSPUO_2$ hydrogel containing solution showed an increase in pH (8.23) due to oxygen generation and release by CSO_2 (chitosan hydrogel loaded with sodium percarbonate but not coated with polyurethane). The maximum increase in pH was observed till day 15. After that, no significant change in pH was noted. It depicts that the oxygen generating ability of SPC was retained for 15 days. To further prove the prolonged and

sustained oxygen generation comparison of polyurethane coated and uncoated hydrogel was carried away. Figure 3B shows the comparison of oxygen release from polyurethane coated ($CSPUO_2$) and uncoated (CSO_2) hydrogel. Uncoated hydrogel (without PU) showed abrupt release of oxygen as the pH of PBS media increased to 10.93 in just 24 hours and 11.20 in 3 days. After that, no increase in pH was observed. While the polyurethane coated hydrogel ($CSPUO_2$) showed a gradual increase in pH till day 15 and the maximum increase in pH was 8.23.

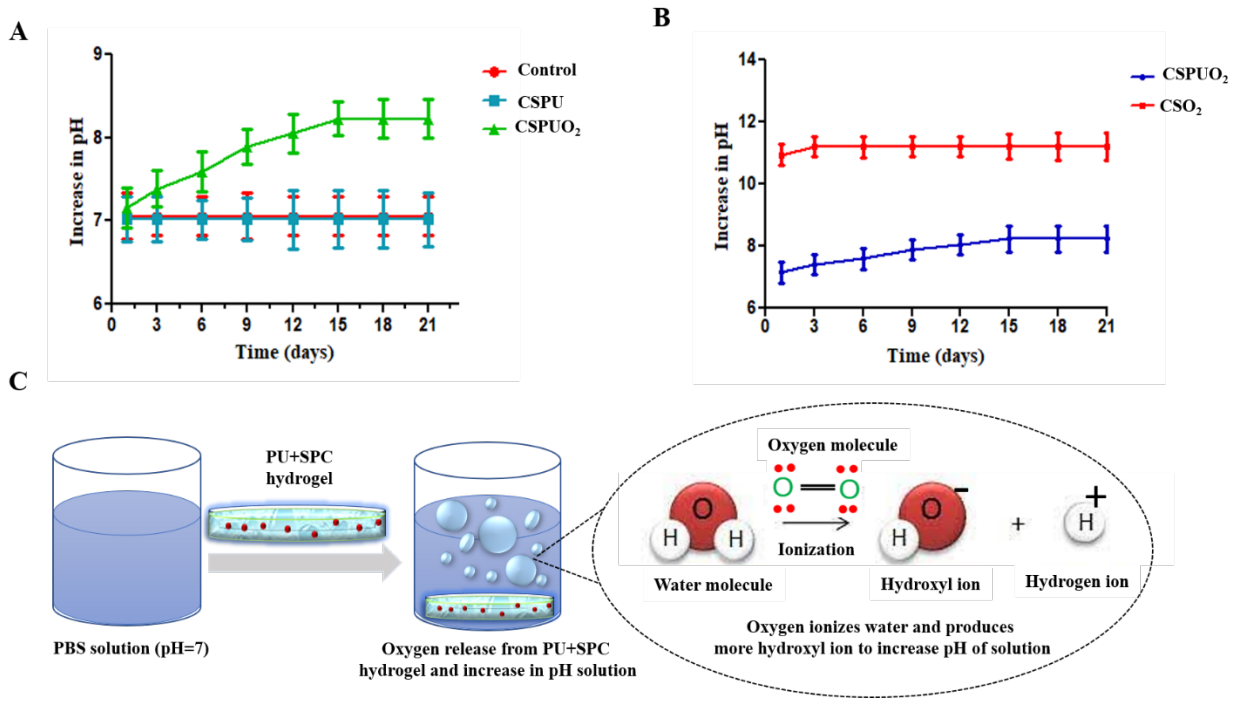

Figure 3. Oxygen generation from sodium per carbonate loaded hydrogel in PBS solution at room temperature without stirring. (A) Oxygen release from $CSPUO_2$ hydrogel while no release from Control and CSPU hydrogel. (B) Prolonged oxygen release from $CSPUO_2$ hydrogel as compared to abrupt oxygen release from CSO_2 (chitosan hydrogel loaded with SPC but not coated with polyurethane) hydrogel. (C) Schematic representation of oxygen release and subsequent pH increase by sodium-per carbonate-based chitosan hydrogel.

3.6. Cytocompatibility studies

3.6.1. MTT assay

The MTT assay was performed to test the cytocompatibility of all three hydrogels. None of the tested hydrogel showed any sign of cytotoxicity. Indeed, cells indicated greater metabolic activity when cultured in indirect contact with CSPU and $CSPUO_2$ as compared to Control and TCP. MTT

assay results at days 3 and 7, demonstrated higher cell viability by hydrogel containing SPC as compared to Control hydrogel. The results suggested that the released oxygen over 7 days gradually accelerated cell proliferation (Figure 4C).

3.6.2. Wound scratch assay

The potential of all the three hydrogels to heal wounds was indirectly tested *in vitro* by looking at their effect on cells proliferation and migration. Significant cell migration was observed in all hydrogels (figure 4A) after 24 hours (around 20 cells migrated in Control, above 30 in CSPU and around 50 in $CSPUO_2$ hydrogel). Quantitative data analysis shows significant difference between $CSPUO_2$ and Control and CSPU hydrogel after 24 hours (Figure 4B).

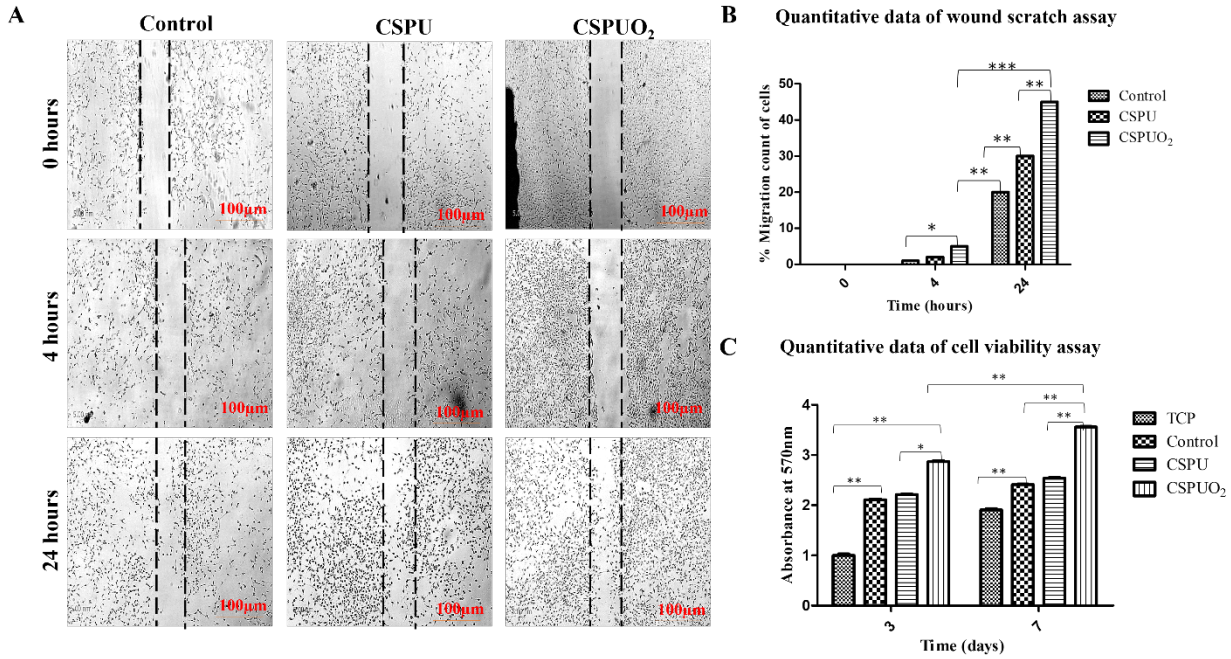

Figure 4. Assessment of migration of cells *in vitro.* (A) Images were taken by microscope showing the migration of cells. (B) Graphical representation of cell migration. One way ANOVA analysis of variance implies a significant difference (*** $P < 0.0001$). (C) The assessment of biological properties of the cells seeded dressings using MTT assay. One way ANOVA analysis of variance implies significant difference (*** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$).

3.7. Analysis of antibacterial potential by turbidimetric assay

Results of the turbidimetric assay indicated that hydrogels possess antibacterial potential (figure 5). Overall, all three hydrogels (control, CSPU and $CSPUO_2$) showed good antibacterial activity against *E. coli*. Control and CSPU hydrogels presented almost similar antibacterial ability against

both strains while $CSPUO_2$ hydrogel showed comparatively greater antibacterial activity than the Control and CSPU hydrogels.

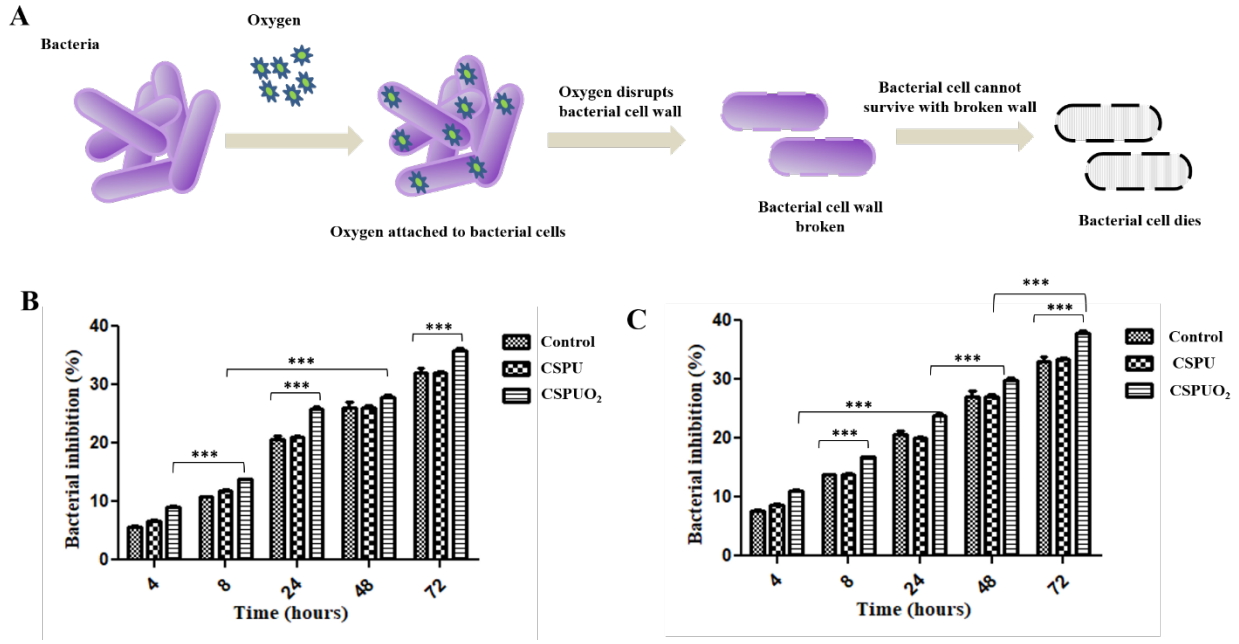

Figure 5. Assessment of the antibacterial potential of hydrogels. (A) schematic representation of oxygen action to kill bacteria. (B) Represents antibacterial activity against S. *aureus*, (C) represents the antibacterial activity of hydrogels against E. *coli*. Graphical data presented as mean (n=3) ± standard deviation of three independent experiments.

3.8. CAM assay

The chick chorioallantoic membrane (CAM) assay was performed to investigate the angiogenic potential of all three hydrogels (Control, CSPU and $CSPUO_2$). It was investigated whether the oxygen generating biomaterial has the potential for neovascularization. Overall, all three hydrogels were covered by thick vasculature and blood vessels were observed passing through ex-planted hydrogels which described the pro-angiogenic nature of these hydrogels (figure 6B). The Control hydrogel was attached to CAM and attracted the blood vessels. Ingrowth of vessels was also observed. In the case of CSPU hydrogel, there was no significant increase in the development of blood vessels as compared to Control hydrogel. But the hydrogel $CSPUO_2$ showed a significant increase in the growth of blood vessels (Figure 6C). The blood vessels were also observed grown into the ex-planted scaffolds. The $CSPUO_2$ hydrogel having sodium per carbonate in it proved to be more chemoattractant for vasculature than Control and CSPU hydrogel as statistical analysis showed significant difference between $CSPUO_2$ and Control, and $CSPUO_2$ and CSPU hydrogel

(figure 6D). This hydrogel was well-attached to CAM and attracted a significantly greater number of blood vessels around as well as inside it.

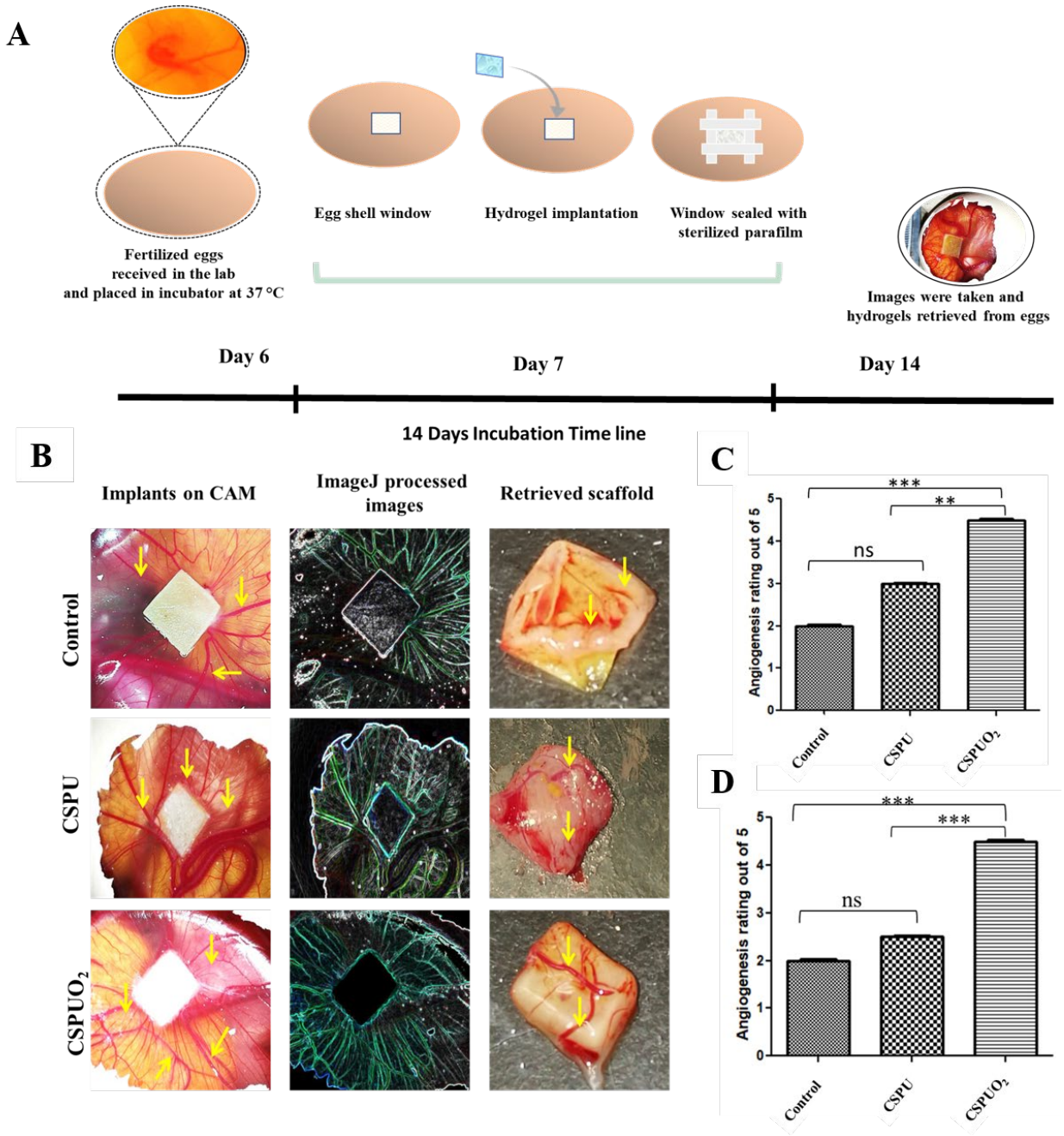

Figure 6: (A) Schematic timeline of CAM assay. (B) Macroscopic appearance of the implants on completion of CAM assay. (C) Quantitative evaluation of a number of blood vessels counted by blind scoring of taken images while performing the CAM assay. (D) Quantitative evaluation of blood vessels counted by blind scoring of retrieved scaffolds. Arbitrary units 1–5 was used to evaluate retrieved scaffolds. Whereas: 1 [zero invasion no blood vessels)], 2 [minute invasion (1-3 number of blood vessels)], 3 [significant invasion (3-5 blood vessels)], 4 [enormous invasion

(5-7 blood vessels)] and 5 [exceptional invasion (more than 7 blood vessels)]. One way ANOVA analysis of variance implies significant difference (*** $P < 0.0001$, ** $P < 0.01$, *$P < 0.05$).

3.9. Animal studies

Wound healing analysis in full thickness wound model was done by macroscopic and percentage wound closure assessment by application of oxygen releasing hydrogel. Figure 7E shows the descriptive macroscopic images of control, CSPU and $CSPUO_2$ experimental group wounds on day 0, 5, 10, 15, 20, 25 and 29. All three hydrogels (control, CSPU and $CSPUO_2$) were effective in wound healing but $CSPUO_2$ hydrogel showed a significant difference in %wound closure. On 5th day %wound closure in the control group was 3.7%, in the CSPU group was 4.9% and in $CSPUO_2$ was 17.35%, similarly, at the 20th day a clear difference in wound closure was observed when %wound closure in control was 41.55%, in CSPU was 54% and in $CSPUO_2$ was 78%. On day 29 $CSPUO_2$ group showed 99.95% wound closure but the control showed 79% and CSPU showed 84% wound closure. Figure7C is showing the percentage wound closure analysis in all experimental groups on day 0, 5, 10, 15, 20, 25 and 29 post transplantation. Though all groups possess significant healing potential and huge difference in %age wound closure was observed in all experimental groups throughout the whole duration.

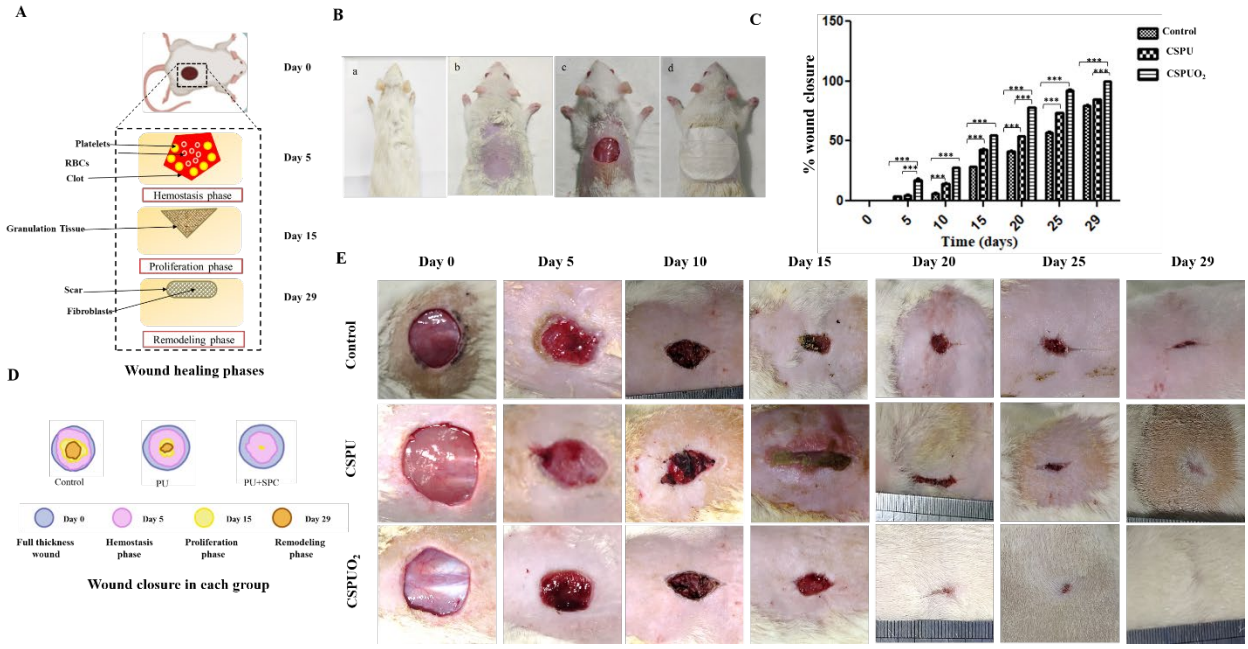

Figure 7. (A) Graphical representation of wound healing phases. (B) Steps of wound making process a. cleaning rat skin, b. shaving of hair, c. full-thickness wound, d. application of treatment. (C) Representative photographs of wounds

on days 0, 5, 10, 15, 20, 25 and 29 post-wounding. (D) Graphical representation of percentage wound closure with respect of time. One way ANOVA analysis of variance implies significant difference (*** P < 0.0001, ** P< 0.01, *P < 0.05). (E) Graphical representation wound closure in each group.

3.10. Histological Evaluation

To appraise the underlying condition of skin tissue during the healing process on the 29th day, the rat's skin samples were collected at the wound site for H&E staining. This detailed histological evaluation is based on the fundamental components of wound healing like formation of neonatal blood vessels, presence of polymorphonuclear inflammatory cells, reconstruction of connective tissue matrix and wound contraction and re-epithelialization. Analysis also revealed that $CSPUO_2$ treatment was more effective in improving the architecture of the epidermis and dermis compared to both CSPU and Control groups indicative of better epithelization (figure 4B). Low number of inflammatory cells in $CSPUO_2$ hydrogel as compared to Control and CSPU hydrogel (figure 4C) and improved vascularization in $CSPUO_2$ hydrogel as compared to Control and CSPU hydrogel also indicate better wound healing capacity of $CSPUO_2$ hydrogel.

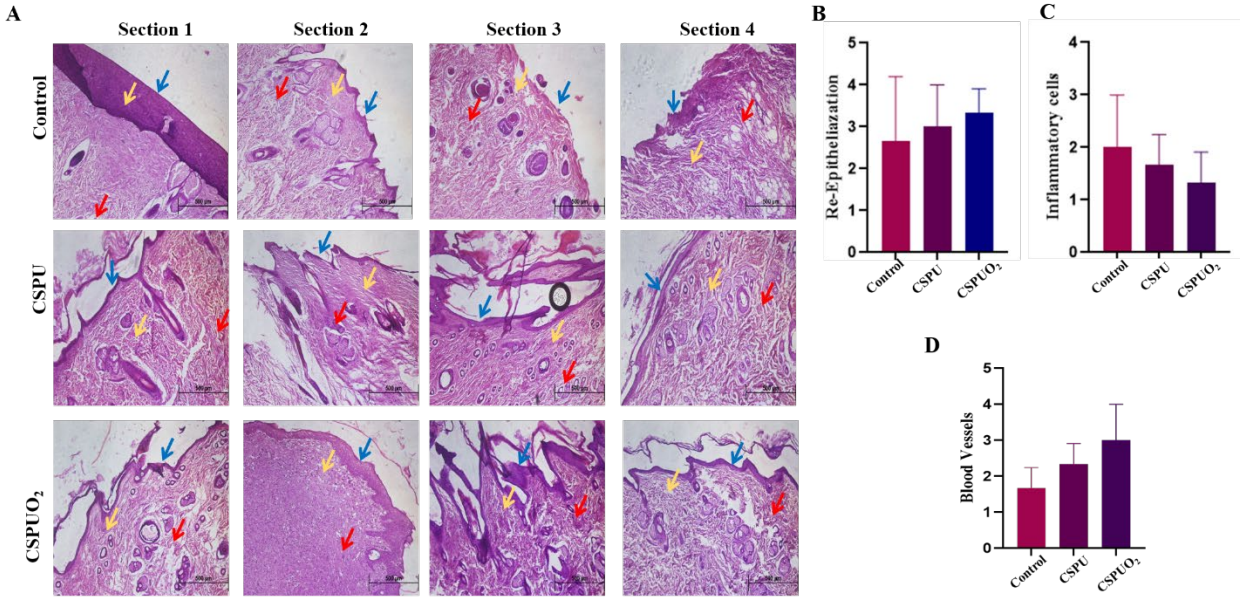

Figure 8. Histological evaluation of wounds treated with hydrogels. (A) Immunohistochemistry-stained images for control, CSPU and $CSPUO_2$ hydrogel at day 29 respectively. (B) Quantitative data of degree of re- epithelialization (C) Quantitative data of inflammatory cells (D) Quantitative data of blood vessels.

4. Discussion

Numerous clinical challenges such as lack of angiogenesis, poor blood circulation, unresolved or prolonged inflammation, and infection are making wound care management a difficult process. A multifunctional wound dressing is required to address the multiple issues of chronic wounds [14]. An ideal wound dressing must be effective in preventing infections and promoting angiogenesis, while simultaneously providing the biocompatibility, biodegradability, high degree of exudate absorption, convenience of application, removal, and minimal frequency of dressing changes [15]. In the present study, we present the synthesis, development, and characterization of a biocompatible and biodegradable hydrogel-based wound dressing loaded with sodium percarbonate as oxygen releasing substance and coated with polyurethane to ensure sustained oxygen release for better angiogenic and antibacterial activities, for vigorous wound healing.

Based on the results (figure 1A) the self-cross-linked hydrogel of chitosan was successfully fabricated with a good porous structure [16] as observed in SEM analysis. Well defined porous structure of a wound dressing ensures the high absorption power to absorb the wound exudates. Heavy wound exudates increase the time of healing as well as the risk of infection and the good dressing must absorb the exudate [17]. Absorption studies result showed the significant absorption capacity (850% of Control, 669% of CSPU, and 621% of $CSPUO_2$) of the hydrogels. Biodegradability is another critical parameter of a wound dressing that depicts the life of biomaterials [18]. We mimic the physiological environment, in this experiment, by using lysozyme (1 mg/mL of PBS) and H_2O_2 (3.5 M H_2O_2 solution in PBS) media. Hydrogen peroxide on dissociation produces highly oxidizing species (hydroxyl radicals) which target the β-d-(1 → 4) glycosidic bonds of chitosan and subsequently break them [19]. Similarly, lysozyme attacks and breaks 1,4-β glycosidic bonds [20]. In degradation studies, after 42 days the control hydrogel showed highest degradation CSPU hydrogel showed a little lower degradation while the $CSPUO_2$ showed the least degradation. Less degradation in CSPU and $CSPUO_2$ hydrogel is due to polyurethane coating, as polyurethane is resistant to degradation [21].

A multifunctional wound dressing needs an active biomaterial to guarantee fast wound healing [22]. We loaded sodium per carbonate as active biomaterial that is reported for its oxygen generating ability and subsequent wound healing potential through angiogenesis, skin regeneration and antibacterial abilities. Sodium per carbonate based wound dressings have been widely reported in literature (table 2). Few oxygen-based dressings for non-infected and lightly exuding chronic

wounds are commercially available like OxyBand™ that is FDA approved oxygen providing wound healing dressing. But the issue is the abrupt release of oxygen. In this study, polyurethane was used to coat the hydrogel to regulate the generation and release of oxygen from sodium per carbonate loaded chitosan hydrogel. Results of the oxygen detection assay (figure 3) showed that oxygen release was gradual and sustained as continuous release was observed till day 15th. A comparison of polyurethane coated and uncoated hydrogel showed the contrasting difference (pH increase to 11.20 in 3 days by uncoated hydrogel vs pH increase to 8.23 in 15 days by coated hydrogel) between oxygen release patterns from these hydrogels. It showed the regulated and sustained oxygen release for 15 days. So, on the wound site oxygen could be provided for 15 days to fight against hypoxia conditions.

Table 2. Reported oxygen generating scaffolds for skin and bone regeneration.

No.	Scaffold	Oxygen generating material	Application	Oxygen release behaviour	Reference
1	Alginate hydrogel	Calcium per oxide	Sub-cutaneous wound healing	48 hours	[23]
2	Gelatin hydrogel	Calcium per oxide	Sub-cutaneous wound healing	12 days	[24]
3	PPGA and HBPL hydrogel	Manganese oxide	Sub-cutaneous wound healing	30 minutes	[25]
4	Poly caprolactone nanofibers	Sodium per carbonate	Sub-cutaneous wound healing	10 days	[26]
5	Polycaprolactone hydrogel	Calcium per oxide	Bone regeneration	14 days	[27]
6	Alginate hydrogel	H_2O_2-PLGA	Sub-cutaneous wound healing	3 days	[28]
7	Biphasic calcium phosphate robocasted scaffold	Calcium per oxide	Bone regeneration	13 days	[29]
8	Chitosan hydrogel	Perfluorocarbon	Sub-cutaneous wound healing	120 hours	[30]

Regulated amount of oxygen is essential at wound site as its high amount may harm the cells by creating the hyperoxia at wound site [31]. The cytotoxicity of hydrogen and oxygen concentration was analyzed by MTT assay. Results demonstrated that there were no adverse effects of these hydrogels or oxygen concentration on fibroblast survival. Indeed, the presence of sodium per carbonate stimulated the metabolic activity of these cells over the period of 7 days. Further, the *in vitro* effect of hydrogel on the skin cells was evaluated through cell migration assay and it was observed that skin cells migrated by the induced oxygen concentration. In case of $CSPUO_2$ hydrogel significant number of cells were migrated after 7 days. The sustained oxygen delivery at wound area was the major aim of the study as killing the bacteria and minimizing the risk of bacterial infection is major requirement in case of chronic wounds. Regulated oxygen delivery at the site of the wound acts as an anti-bacterial agent [32]. Results of antibacterial assay proved that $CSPUO_2$ hydrogel showed great capacity to inhibit both *E. coli* and *S. aureus* bacterial strains (37% and 35% inhibition rate respectively) after 72 hours that are frequently responsible to cause infection in wounded areas. The inhibition rate may further increase with the passage of time as with time more oxygen is released from the hydrogel to inhibit the growth of microbes with significant inhibition rate [33, 34]. Control and CSPU hydrogel also showed good potential to inhibit bacterial growth due to chitosan as it possesses significant anti-bacterial potential and even reported for its ability to kill drug resistant bacteria [35]. The CSPU hydrogel presented good antibacterial activity depicting PU coating did not affect the antibacterial potential of chitosan.

Damaged blood vessels and poor blood supply are principal issues of chronic and burn wounds and a multifunctional wound healing dressing must address this issue [36]. The angiogenic potential of synthesized hydrogels was explored by the chick chorioallantoic membrane (CAM) assay. Results proved that all hydrogels have significant potential to develop new blood vessels. But $CSPUO_2$ hydrogel presented the greatest angiogenic potential as compared to control and CSPU hydrogel. The thick network of blood vessels and significantly different results of $CSPUO_2$ hydrogel as compared to control hydrogel proved that sodium per carbonate has promoted angiogenesis and this property was attributed to the generation of ROS (H_2O_2) and molecular oxygen.

Compromised wound healing is a life-threatening issue along with the principal cause of many associated health problems [37]. The present study ultimately intended to investigate the healing effect of released oxygen from chitosan hydrogel in a full thickness wounded rat model. Results

showed that all three hydrogels (Control, CSPU and $CSPUO_2$) were effective in wound healing but $CSPUO_2$ hydrogel showed a significant difference in %wound closure as on final day $CSPUO_2$ group showed 99.95% wound closure but the control showed 79% and CSPU showed 84% wound closure. Results of H&E staining also showed that the $CSPUO_2$ group improved the skin texture and re-epithelization was better in this group as compared to CSPU and control group.

Histological evaluation further showed poor granulation, partial healing, improper epithelium formation, and fewer blood vessels in the control group; haemorrhage, necrosis, and scab were also still present in the CSPU and control group (Figure 8A). In the CSPU and $CSPUO_2$ groups, the healing was significantly enhanced, the epithelialization rate was higher, and the granulation tissue and capillary formation were increased. Interestingly, less inflammatory permeation in $CSPUO_2$ group was found as compared to control group where there is clear permeation of polymorphonuclear and mononuclear inflammatory cells, the proposed reason behind this difference is the role of SPC to survive cells under hypoxic conditions and decrease rate of apoptosis and necrosis through constant production of oxygen [38]. No inflammatory response was observed in the treated animals and Liang, Y., et al have reported that no inflammatory response is an indication of biocompatibility of applied treatment [39]. Moreover, $CSPUO_2$ showed immense neovascularization as compared to only CSPU and Control group which is clear indication of proper wound healing properties of SPC which are again contributed to its oxygen production which is vital for the maturation of skin tissues (Figure 8B). The skin defect in the $CSPUO_2$ group recovered more quickly than that of the other two groups, and we even noticed some new hair follicle structures. Overall, the $CSPUO_2$ hydrogel in SD rats' skin wounds demonstrated the promising therapeutic effect.

The study presents an efficient approach of sustained oxygen delivery to treat diabetic and burn wounds. By releasing sustained amount of oxygen, this dressing ($CSPUO_2$) ensures good angiogenic and antibacterial properties for fast wound healing. The designed approach also provides multiple benefits i.e., absorption capacity, biodegradability abs compatible to skin. It can be a multifunctional dressing for heavily exudating wounds at affordable price.

5. Conclusion

In summary, in this study we have developed a polyurethane coated sodium per carbonate loaded chitosan hydrogel as a multifunctional wound dressing. The most attractive feature of the this developed hydrogel is sustained oxygen delivery at wounds, which ensured anti-bacterial and

neovascularization ability better than the hydrogels not having sodium per carbonate and polyurethane coating. In full thickness wound model, the fabricated hydrogel not only enhanced the rate of wound healing but also enhanced the re-modeling of the healed skin. Histological evaluation ensured tremendous skin re-epithelialization and tissue granulation ability. Overall, the developed approach provides a multifunctional wound dressing to address multiple issues of chronic wounds. In addition, due to the simple process of production and application, the developed hydrogel might have a great potential of being applied in clinical settings to treat burn and diabetic wounds.

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