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Bioactive polymeric formulations for wound healing

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Abstract

Ricinoleic acid (RA) has potential to promote wound healing because of its analgesic and anti-inflammatory properties. This study investigates the synthesis and characterization of RA liposomes infused in a hydrogel for topical application. Lecithin liposomes containing RA were prepared and incorporated into a chitosan solution and were subsequently cross-linked with dialdehyde β -cyclodextrin (Di- β -CD). Chitosan/Di- β -CD concentrations and reaction temperatures were varied to alter gelation time, water content, and mechanical properties of the hydrogel in an effort to obtain a wide range of RA release profiles. Hydrogel cross-linking was confirmed by spectroscopy, and liposome and carrier hydrogel morphology via microscopy. Chitosan, Di- β -CD, and liposome concentrations within the formulation affected the extent of matrix swelling, mechanical strength, and pore and overall morphology. Higher cross-linking density of the hydrogel led to lower water uptake and slower release rate of RA. Optimized formulations resulted in a burst release of RA followed by a steady release pattern accounting for 80% of the encapsulated RA over a period of 48 hours. However, RA concentrations above 0.1 mg/mL were found to be cytotoxic to fibroblast cultures in vitro because of the oily nature of RA. These formulations promoted wound healing when used to treat full thickness skin wounds (2 cm²) in Wister male rats. The wound contraction rates were significantly higher compared to a commercially available topical cream after a time period of 21 days. Histopathological analysis of the RA-liposomal chitosan hydrogel group showed that the epidermis, dermis, and subcutaneous skin layers displayed an accelerated yet normal healing compared to control group.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords

chitosan hydrogel; liposomes; ricinoleic acid; topical application; wound healing

1 | INTRODUCTION

Over 6 million cases of chronic wounds are reported in the USA alone each year with treatment costs exceeding 25 billion dollars annually.¹ Full thickness wounds extend beyond the epidermis and dermis and affect blood vessels, sweat glands, and hair follicles.² Current treatment strategies include application of creams containing synthetic drugs, antibiotics, and growth factors to promote wound healing.¹ Effective wound management requires repeated application of these creams as well as wound coverage to manage drug bioactivity, moisture, and sterility at the wound site.² Both industry and academic research is focused on the development of novel technologies and strategies for wound care to reduce the overall medical expenses.

Current research efforts are focused on the development of novel bioactive formulations as alternatives to traditionally used antibiotics and growth factors to promote wound healing and to lower medical expenditures.² The analgesic and anti-inflammatory properties of natural products, such as ricinoleic acid (RA) and capsaicin, make them attractive for wound healing applications.³ However, capsaicin-based formulations present several drawbacks including pungent odor, skin irritation, and hyperalgesic pain, making them less attractive for wound healing applications.^{4,5} RA extracted from castor oil may provide an alternative to capsaicin,^{3,6} if formulations can avoid oxidative RA rancidity and provide efficient permeation through the skin.⁷

Drug delivery systems such as emulsions,^{8–10} pastes,¹¹ microparticles,¹² nanoparticles,¹³ polymeric barrier membranes,^{14,15} nanofiber matrices,^{16,17} and hydrogels¹⁸ are commonly used in topical applications. However, permeation of bioactive molecules across the lipophilic skin layers still remains a major challenge in these formulations.¹⁹ Phospholipid-based liposomal formulations have the ability to pass through the lipophilic layers of skin and facilitate drug penetration deep into the stratum corneum.^{20–23} Phospholipids are bilayer membranes that assemble into vesicles and liposomes, based on the lipid composition and concentration.^{24–26} These carriers can be used to encapsulate both hydrophilic and hydrophobic bioactive molecules into a hydrophobic bilayer vesicle or core of the liposome. However, liposomal formulations over time lose their size and shape to form larger aggregates with limited permeation rates across the skin.^{15,26} Aggregation of liposomes can be avoided by presenting them in the form of a solution or in a carrier hydrogel.²⁷ Hydrogel-infused liposomal formulations are easier to apply on the wound site and provide localized, controlled release.²⁸

A variety of hydrogels have been developed using both natural and synthetic polymers via chemical cross-linking or physical chain entanglements to produce a water-insoluble cross-linked polymer network.^{29–31} Polysaccharides such as chitosan,³² β -cyclodextrin,³³ cellulose,³⁴ starch,³⁵ alginate,³⁶ and hyaluronic acid³⁷ based hydrogels have been used for the delivery of several bioactive molecules. The drug release rate from these carriers greatly

depends on the crosslinking density, because it regulates water uptake, mechanical strength, and pore properties of the hydrogel.³⁸ Additionally, hydrogels provide a non-adherent bandage at the wound site while maintaining the moisture to promote wound closure.³⁹

Chitosan is a deacetylated product of chitin obtained from the cell walls of seaweed and exoskeletons of crustaceans.³⁹ It is a linear animated polysaccharide and is used in a variety of drug delivery and tissue engineering applications because of its excellent biocompatibility and versatile material properties.⁴⁰ Its antimicrobial property and ability to retain moisture at the wound site makes chitosan attractive for wound healing dressings.^{40–42} Chitosan-based drug delivery and scaffold systems are cross-linked with a variety of cross-linking agents to control their drug release pattern and structure in a biological environment.⁴³ The choice of cross-linking agent often determines the toxicity and biological outcome. Efforts are being made to replace traditionally used cross-linking agents such as glutaraldehyde, formaldehyde, epoxides, and other synthetic acrylic monomers with biocompatible cross-linking agents.⁴³ Previously, we have reported the use of dextran dialdehyde as a crosslinking agent for chitosan and showed its safety and efficacy in cell delivery and tissue engineering applications.⁴⁴ Amine functional groups of chitosan were cross-linked through a reaction with aldehyde group of dextran dialdehyde resulting in an imine bond (Schiff's base) formation.^{43,44}

We report on the synthesis and characterization of a new formulation comprised of RA liposome infused in cross-linked chitosan and its efficacy on wound healing. A dialdehyde cross-linker was synthesized using β -cyclodextrin according to our published protocol.^{44,45} Polymers and formulations were characterized for structure, morphology, mechanical strength, water uptake, and RA release pattern using various analytical techniques. Cell compatibility of the hydrogel formulations and RA was evaluated using in vitro cell culture experiments. Full thickness wound defects in male Wistar rats were treated with chitosan hydrogel formulations to evaluate wound contraction rate and healing.

2 | EXPERIMENTAL

2.1 | Materials

Lecithin granule (DAS gesunde PLUS) derived from natural soya and USP grade castor oil (Home Health) were obtained from Slovakia. Chloroform, ethylene glycol, ethyl acetate (ETAC), potassium hydroxide (KOH), sodium hydroxide (NaOH), hydrochloric acid (HCl), acetic acid, sodium meta periodate, sodium borohydride, chitosan, β -cyclodextrin, and deuterated dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). Murine 3T3 fibroblasts were purchased from DSMZ (Braunschweig, Germany). Dulbecco's minimum essential medium, fetal bovine serum, antibiotics (penicillin, streptomycin P/S), and trypsin-EDTA were purchased from Life Technologies (Grand Island, NY). Fluorescein dye was purchased from ACROS Organics (Fisher Scientific). All other reagents and materials were used without further purification. All other chemicals used in this study were purchased from Fisher Scientific or Aldrich Chemicals and were used without further purification.

2.2 | Synthesis of ricinoleic acid

Ricinoleic acid was extracted from castor oil using alkaline ethanol followed by neutralization with an acid.⁴⁶ In brief, 50 g of dried castor oil and 200 mL of ethanol with 12 g of KOH were charged into a 500-mL round-bottom flask fitted with a water condenser and refluxed at 150°C for an hour. This reaction mixture was concentrated using a rotary evaporator and poured into 200 mL deionized water followed by neutralization with HCl to obtain an oily layer. The oil was extracted in 600 mL ETAC using a separatory funnel and washed repeatedly (3×) with hot water and NaCl then dried overnight using magnesium sulfate. This extract was filtered and ETAC dried at 50°C using rotary evaporator. The final product (yield 86%) was kept refrigerated until further use.

2.3 | Preparation of di-aldehyde β -cyclodextrin

The controlled β -cyclodextrin oxidation mediated by sodium metaperiodate (NaIO_4) to obtain di-aldehyde β -cyclodextrin (Di- β -CD) is presented in Figure S3.⁴⁷ In brief, a 5 wt.% aqueous solution of β -CD in a beaker was adjusted to pH 4 and stirred on an ice bath, and aqueous NaIO_4 was added dropwise at a molar ratio of β -CD/ NaIO_4 = 1:3. The reaction mixture was stirred for 2 hours at 0°C and left overnight at room temperature. Oxidation was quenched by adding a few drops of ethylene glycol. The final product was freeze-dried to obtain Di- β -CD and stored at 4°C in dark until further use.⁴⁸

2.4 | Preparation of ricinoleic acid-liposome formulations infused in chitosan

Liposomal formulations containing RA were prepared by reverse phase evaporation method.⁴⁹ In brief, 2 g lecithin and 1.5 g of RA were dissolved in 50 mL chloroform in a 1-L round-bottom flask. This solution was stirred for 30 minutes at room temperature and dried using a rotary evaporator. Following solvent removal, a thin layer of lipid bilayer containing RA was formed inside the flask surface. Figure S4 summarizes the schematics of the liposome formulation fabrication process. An aqueous chitosan solution at 1, 2, and 3 wt.% was prepared using 1% acetic acid, and 50 mL of chitosan solution was added to the liposome RA mixture with constant stirring at 1500 rpm at 60°C for 3 hours to obtain a white suspension. This suspension was concentrated using rotary evaporator at 50°C for 2 hours to obtain a gel-like consistency. The gel was diluted in deionized water to 60 mL followed by sonication for 2 hours in an ultrasonic bath (Fisher Scientific, FS-21H) and for 15 minutes using ultrasonic probe (Misonix, Sonicator 3000). The final liposomal formulation was kept refrigerated until further use.

2.5 | Preparation of di-aldehyde β -cyclodextrin cross-linked chitosan formulations containing ricinoleic acid liposomes

Freshly prepared chitosan solution containing RA liposomes (10 mL) was mixed with 1 mL of Di- β -CD. The solution was maintained at 70°C with vigorous mixing for 10 to 15 minutes to cross-link chitosan. In this study, 3 different concentrations of Di- β -CD namely 5, 7.5, and 10 wt.% were used to optimize the cross-linking density. Similarly, 3 different chitosan concentrations 1, 2, and 3 wt.% were used to alter carrier gel concentrations. Cross-linked hydrogel cake formulation was transferred to a histology cassette and placed in a reduction buffer at pH 4 containing excess of sodium borohydride for 24 hours to generate

stable secondary amine. The hydrogel formulation containing RA liposomes was washed repeatedly with DI water, freeze-dried, and stored in desiccator until further use.

2.6 | Characterization of hydrogels

2.6.1 | Spectral analysis—FTIR spectral measurements were performed using a Nicolet (Thermo Scientific, Nicolet iS10, USA) spectrophotometer to confirm the RA structure and cross-linking reaction between chitosan and Di- β -CD. RA structure was confirmed using ^1H NMR spectrometer, Avance III 500 (Bruker Biospin, Germany). Solutions were prepared in deuterated dimethyl sulfoxide solvent, and tetramethylsilane was used as an internal standard.

2.6.2 | Hydrogel and liposome imaging—The surface and cross-sectional morphology of both chitosan-Di- β -CD hydrogel and RA-liposomal chitosan hydrogel following freeze drying was examined by scanning electron microscopy (SEM) using JEOL JSM-6335F (JEOL, Boston, MA, USA) at various magnifications. The polymer surfaces were sputter coated with Au/Pd using a Hummer V sputtering system (Technics, Baltimore, MD, USA) before visualizing under SEM. The microstructure of the liposome, stained with fluorescein dye, was assessed using the Leica DM2000 fluorescence light microscopes with microscope software LAS X.

2.6.3 | Swelling studies—The extent of water absorption by chitosan-liposomal formulations with and without RA was carried out by incubating 100 mg dry thick membrane samples in PBS buffer pH 7.4 at 37°C. Initial dry weights of the formulations were recorded as W_a and equilibrium swelling weight as W_b . All these measurements were carried out in triplicate. The swelling ratio was expressed as in Equation 1:

$$\% \text{Swelling ratio} = \frac{(W_b - W_a)}{W_a} \times 100 \quad (1)$$

2.6.4 | Mechanical properties and the gelation time—The oscillatory shear measurements of storage modulus of chitosan formulations with and without RA liposomes were measured at room temperature using constant stress rheometer (TA Instruments Discovery Hybrid Rheometer 3 DHR-3) with parallel plates and spindle diameter 12 mm. In brief, 200 μL of chitosan formulation with cross-linker were placed on the lower plate of the rheometer, and the upper plate was lowered to gap size 700 μm immediately. The storage (G') and loss (G'') moduli were recorded using an amplitude sweep from 0.001% to 10% strain.

Gelation time for the formulations at the different chitosan and cross-linking agent concentrations was recorded based on the time required to form a solid mass for visual observations. Formulations were placed in an Eppendorf tube and agitated in a thermal rocker incubator at various temperatures for required time for the solution solidification. These studies were conducted in triplicates for each formulation and presented as avg. $\pm$ std. dev.

2.7 | Encapsulation efficiency

Ricinoleic acid content in the formulations was estimated by extracting RA into 7.4 pH phosphate buffer with methanol and acetone. The samples were then filtered, and absorbance was recorded at λ_{max} of 222 nm. The encapsulation efficiency was then calculated using Equation 2:

$$EE\% = \frac{\text{Practical loading drug}}{\text{Theoretical 1 loading drug}} \times 100 \quad (2)$$

2.8 | In vitro drug release study

The total release of free RA and liposome encapsulated RA from the chitosan liposome formulation was carried out by incubating 50 mg of the freeze-dried samples in 10 mL of PBS (pH 7.4) at 37°C in a shaker water bath. At regular time intervals, 1 mL of the release media was taken out and replenished with equal amount of PBS.⁴³ At time intervals, 1 mL of the released medium was taken and 1 mL methanol was added to lyse the liposomes and 2 mL acetone was added to dissolve RA. The 4 mL mixture was filtered through 0.2- μm membrane, and the absorbance readings of the supernatant were recorded at 222 nm using UV-Vis spectrophotometer (SHIMADZU, UVmini-1240). The total amount of free RA released was calculated from a standard calibration curve. The amount of RA released was calculated using a standard calibration curve and plotted as a cumulative release.⁵⁰

2.9 | Cytotoxicity test

All cytotoxicity studies were performed on murine 3T3 fibroblasts at passage 6. Culture media Dulbecco's minimum essential medium was supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and 1% antibiotics (streptomycin/penicillin), and the cells were maintained in a humidified tissue culture incubator at 37°C and 5% CO_2 . Each well in a 96-well plate was seeded with 5000 cells for assessing the cytotoxicity of the leachates from the formulations. Formulations were sterilized under ultraviolet (UV) light for 30 minutes in a laminar flow before extraction, and the extracted media were filter-sterilized using a 0.22- μm syringe filter. Cells were treated with extracted RA at concentrations ranging from 0.1 to 100 mg/mL, and cytotoxicity was measured after 24 hours. All treatments and measurements were performed in quadruplicates. Cell viability was determined using MTT colorimetric assay kit according to the manufacturer's instructions.⁵¹ In brief, 100 μL growth media with MTT reagent (0.5 mg/mL) was added and incubated for 2 hours. Resulting product that was produced by the cells was extracted in 100 μL DMSO, and absorbance was read at the wavelength 595 nm using a Biotek (Winooski, VT) plate reader.

2.10 | Evaluation of wound contraction rate in rat skin defect model

The animal study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the National Research Center (NRC), Dokki, Giza. Surgeries were performed on male Wister rats (120 g, NRC, Dokki, Giza)⁵² following standard aseptic procedures. Rats were housed in separate cages with access to food and water. Excision wounds were used to study the wound contraction.

Two full thickness wounds extending up to adipose tissue measuring 2 cm² were created on the back of each rat and was used to evaluate wound contraction rate.⁵³ A total of 12 rats were randomly divided into 4 experimental groups. The experimental groups are as follows.

Group 1-. Vaseline gauze fabric (control, n = 3).

Group 2-. Garamycin commercial cream (control, n = 3).

Group 3-. Chitosan hydrogel formulation infused with RA-free liposomes (control, n = 3).

Group 4-. Chitosan hydrogel formulation infused with RA liposomes (Test, n = 3).

2.11 | Statistical analysis

All results were evaluated using 1-way analysis of variation (ANOVA) followed by Tukey's honestly significant difference analysis of the differences between groups with a confidence range of 95%.

3 | RESULTS AND DISCUSSION

3.1 | Liposome morphology

Liposomal formulations were incorporated with a fluorescein dye to visualize the structure and size of the liposome. Fluorescein dye was incorporated into the liposomes during the fabrication process by mixing it with other excipients. The structure of the bilayer liposome is presented in Figure 1A. These spherical, bilayer structures showed diameters in the range of 42 to 175 μm . Figure 1B illustrates the schematic diagram for the cross-linking reaction between Di- β -CD and the chitosan chains.^{43,44}

3.2 | Synthesis of di-aldehyde β -cyclodextrin

In the FTIR spectra (see supporting information, Figure S5) of β -CD, characteristic bands at 3318, 2925, 1156, and 1027 cm^{-1} represent symmetric and asymmetric vibrations of —OH , —CH_2 , —C—O—C , and the bending vibration of —OH , respectively. The Di- β -CD obtained by selective oxidation of CD has characteristic aldehyde carbonyl bands at 1715 and 766 cm^{-1} confirming the formation of hemiacetal bonds between adjacent hydroxyl groups.

3.3 | Synthesis of ricinoleic acid-liposome infused cross-linked chitosan hydrogels with di-aldehyde β -cyclodextrin

Figure 2A shows the FTIR spectra of di-aldehyde cross-linked chitosan and β -cyclodextrin. The characteristic bands of chitosan are found at 3345 cm^{-1} (amine), 2878 cm^{-1} (—CH_2), and 1022/1153 cm^{-1} (—C—O—C). Following cross-linking, chitosan exhibits a new band at 1634 cm^{-1} (—C=N). The new band confirms the formation of Schiff's base.^{43,44} In the reduced hydrogel, the —C=N band disappeared with a new band —CH—NH— shifted to 1645 cm^{-1} . Additionally, reduction of Schiff base generated a secondary amine group at 3352 cm^{-1} .⁵⁴ Figure 2B illustrates the schematic diagram for RA-liposome-loaded chitosan-Di- β -CD hydrogels. FT-IR spectrum of synthesized RA is found in Figures S1 and S2.

3.4 | Effect of chitosan/di-aldehyde β -cyclodextrin composition on gelation time

Hydrogel formulations are often optimized for their gelation time and cross-linking density to deliver bioactive molecules at the desired location and to achieve release patterns. The cross-linking mechanism of chitosan with Di- β -CD and glutaraldehyde are quite similar.⁵⁵ However, Di- β -CD provides 7 times more aldehyde groups than glutaraldehyde (14 functional groups vs. 2) and therefore may serve as a better cross-linking agent.⁵⁶ The cross-linking mechanism is a slower process at room temperature, and gelation occurs at a rapid rate at higher temperatures. Table 1 summarizes the gelation time for various formulations produced using 3 different chitosan solution concentrations and cross-linking agent at 25°C, 37°C, and 60°C. In general, a shorter gelation time was observed with increasing concentrations of both chitosan and cross-linking agent. Likewise, a shorter gelation time was also observed as reaction temperature increased, because of a greater probability of interactions between the amine and aldehyde groups on different polymer chains with increased mobility and reactivity at higher temperatures.⁵⁵ However, 1 wt.% chitosan solutions failed to produce gels even at higher cross-linking concentration and temperature because of reduced number of imine bond formation with different polymer chains. This was also true for the formulation with 2 wt.% chitosan and least amount of Di- β -CD even after 24 hours.

3.5 | Morphology of di-aldehyde β -cyclodextrin chitosan liposomal hydrogel

Formulations exhibited different morphology based on their composition as evidenced by SEM images.^{43,44} The morphology of the carrier hydrogel and the same gel infused with liposomes produced at 1:0.07 molar ratio of chitosan and Di- β -CD is shown in Figure 3A. Formulations with 3 wt.% chitosan produced dense architecture with a rough surface and small pores. On the other hand, 2 wt.% chitosan produced less dense and bigger pores with relatively smooth surface. Hydrogel formulations with RA liposomes showed smoother surfaces, and pore architecture was absent. The pores in these formulations may have been filled with RA liposomes, thus providing smoother surfaces.^{43,44}

3.6 | Mechanical properties

Mechanical properties are important for deciding an ideal hydrogel formulation for a particular biomedical application. A wide range of mechanical properties was obtained by altering the formulation composition of chitosan and cross-linking agent concentration using a rheometer as presented in Figure 3B. Among all the formulations studied, the 3 wt.% chitosan formulations showed the highest storage modulus because of increased density. There was no significant difference in mechanical properties of the hydrogels when RA-loaded liposomes were incorporated. Formulations with 2 wt.% of chitosan showed a reduction in storage modulus after incorporation of RA liposomes. This decrease in storage modulus may be because of the presence of liposomes between chitosan chains resulting in plasticization and a reduced cross-linking density.⁵⁷ Formulations of 1 wt.% chitosan with varied concentrations of cross-linking agent resulted in the lowest storage modulus, which was expected because of a reduced matrix density and cross-linking.

3.7 | Cytotoxicity test

Cytocompatibility of the chitosan formulations containing RA liposomes was tested in vitro using 3T3 fibroblasts. Figure 4A shows the percentage of viable cells following treatment with leachates chitosan, lecithin, and RA formulations. Cells cultured in regular growth media that were considered 100% viable served as the control group, and test groups were presented as a viable fraction of the control. Leachates of chitosan did not show notable cytotoxicity, and cell viability and cell morphology were similar to untreated control cells. RA at a concentration of 1 mg/mL severely affected the cell viability suggesting this dose to be toxic to the 3T3 fibroblasts. The LC_{50} value for RA falls between 0.1 and 1 mg/mL (Figure 4B). A comparison of cell viability following exposure to leachates of chitosan and chitosan-RA liposomes at various concentrations is presented in Figure 4C. Crosslinked chitosan hydrogel leachate at all concentrations is well tolerated by the cells indicating the carrier hydrogel cell compatibility. Conversely, leachates of the same carrier gel with RA-liposomal formulations were severely toxic to cells. Dilution of the leachates led to better cell viability, and the 1% dilution was comparable to the 1% carrier gel and the control cells. These results are consistent with our earlier report, where chitosan liposomal RA-loaded microsphere formulations at a concentration of 50 mg/mL decreased the viability of human HFB4 fibroblasts to 78% compared to controls.⁵⁸ Hydrogel formulations can be adjusted to provide therapeutic doses of RA by altering the liposomal formulation content.

The cell morphological changes because of RA leachates were evaluated microscopically. Changes in cell morphology are presented in Figure. 4D. Cell morphologies were identical in the groups exposed to leachates of chitosan (Figure 4D (B)) and cross-linked chitosan hydrogel (Figure 4D (E)) compared to control cells (Figure 4D (A)). These observations are similar to cell viability and confirm the cytocompatibility of the carrier hydrogel. However, significant morphological changes were observed when treated with leachates of lecithin (Figure 4D (C)), RA (Figure 4D (D)) and carrier gel infused with RA liposomes (Figure 4D (F)) where cells often detached and assumed a rounded morphology. The cell detachment and rounded morphology was presumably because of decreased attachment to culture plate because of the presence of an oily substance.⁵⁹

3.8 | Swelling property of hydrogels

The degree of swelling determines both the rate of bioactive agent release and the dimensions of the hydrogel in a biomedical application. Swelling significantly influences mechanical integrity and surface properties. Figure 5A–D summarizes the degree of swelling for chitosan formulations with and without RA liposomes. Different formulations were subjected to swelling studies at room temperature at pH 7.4 and 5.7. In general, all of the formulations showed significant water uptake that was higher in pH 5.7, as compared to pH 7.4, because of the increased amine group protonation at lower pH.⁶⁰ Similarly, formulations with RA liposomes showed higher water uptake at both pH ranges as compared to formulations without liposomes. This may be because of the presence of a liposomal phase in the polymer network and the plasticization effect. Formulations of 3 wt.% chitosan with the highest cross-linking concentration showed the lowest degree of swelling. This is a result of higher cross-linking density leading to reduced capability of water absorption of the

hydrogel. For most hydrogels, the swelling degree showed only moderate changes after prolonged incubation from 1 to 24 hours.

3.9 | Encapsulation efficiency and ricinoleic acid release profile

The encapsulation efficiency of RA-liposomal chitosan hydrogel, RA-liposomal chitosan hydrogel/primary amine (PA)- β -CD (0.014:1), RA-liposomal chitosan hydrogel/PA- β -CD (0.04:1), and RA-liposomal chitosan hydrogel/PA- β -CD (0.07:1) was found to be in the range of 90% to 95%. Figure 5E presents the RA release profiles of various formulations with different cross-linking densities over a period of 48 hours at pH 7.4. PA groups obtained, following reduction of chitosan cross-linked with Di- β -CD, provides a stable cross-link, and these formulations are referred as PA- β -CD. All the release studies were carried out with formulations prepared using 3 wt.% cross-linked chitosan either with Di- β -CD or PA- β -CD in an effort to alter the RA release profile. Carrier hydrogels without cross-linking released 80% of RA in the first 15 minutes, while PA- β -CD cross-links released the same amount over a period of 48 hours. Increasing the concentration of PA- β -CD in the formulation led to a higher and more stable crosslinking of the hydrogel network and subsequently a slower release of RA. These results are consistent with many other studies where increased matrix cross-link densities led to slower rates of drug release.^{61–63}

3.10 | Evaluation of wound contraction rate in rat skin defect model

Figure 6A summarizes the wound closure rate following treatment with various chitosan formulations over a period of 21 days. Wounds treated with RA-hydrogel formulations showed a normal appearance of healing comparable to commercial cream. Daily administration of the formulation did not induce extensive acute inflammatory responses or cause any visible erythema. Chitosan formulations with RA liposomes showed superior wound contraction rates compared to other treatment groups. The chitosan formulations with RA were well tolerated as they are non-irritants and do not impact inflammation at the wound site.⁵⁸ Figure 6B shows a (i) wound area of 2 cm² on the Wister rat's skin before treatment with hydrogel and (ii) shows a wound area of 0.04 cm² after treatment (21 days) with the hydrogel. These gel formulations with RA appear to be effective in promoting wound healing.

Figure 6C shows the histologic images of the wound healing process for the different treatment groups. Group 1 is normal skin (control) that is clearly marked by different skin layers including epidermis, dermis, subcutaneous tissue, and musculature. The epidermal layer of group 2 treated with the commercial cream showed focal acanthosis in the prickle cell layer. The underlying dermis of group 2 had granulation tissue formation by fibroblastic cell proliferation as well as newly formed capillaries. The subcutaneous tissue was infiltrated by a massive number of inflammatory cells. Group 3 images treated with chitosan formulation without RA showed similarities with group 2 in the presence of focal acanthosis in the epidermis and new granulation tissue in the underlying dermis; however, there were fewer inflammatory cells infiltrating the subcutaneous tissue. The test formulation containing RA liposomes incorporated in chitosan hydrogel (group 4) showed clearly marked epidermis, dermis, and subcutaneous tissue quite similar to control skin (group 1). Histological findings supported the observations made with respect to wound contraction.

Table 2 summarizes the histopathological investigation of the treated samples of groups 1, 2, 3, and 4 which can be visualized in Figure 6C. Group 1 and 4 data show no histopathological alterations in the histological structure of the epidermis, dermis, subcutaneous tissue, and musculature. This reflects the ability of the RA-liposomal chitosan hydrogel to promote the healing process of wounds. This also supports the superiority of the RA hydrogel compared to the commercial cream. Group 3, treated with the commercial cream, showed little inflammatory cell infiltration and focal acanthosis.

4 | CONCLUSIONS

This study successfully demonstrated the isolation and characterization of RA from castor oil. The application of RA as an anti-inflammatory agent for wound healing application by encapsulating it into liposomes and carrier chitosan hydrogel was explored. A Di- β -CD was used as a biocompatible cross-linking agent and found to be efficient in chitosan cross-linking. The extent of chitosan concentration and cross-linking with Di- β -CD dictated the swelling, mechanical strength, pore size, and morphology of the formulation. Higher crosslinking density led to slower release of RA from the formulations. The test formulations were effective in releasing 80% of the encapsulated RA over a period of 48 hours. However, higher concentrations of RA beyond 0.1 mg/mL appear to be cytotoxic in vitro evidenced by cell lifting and rounded morphology. The carrier gel and the RA-liposomal chitosan hydrogel formulations showed superior contraction rates of the wound area compared to other treatment. Histopathological analysis proved that the epidermis, dermis, and subcutaneous tissues were histologically intact and showed normal healing of the rat skin with RA-liposomal chitosan hydrogel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

(A) Florescence microscope images for the encapsulated RA inside the bilayer of liposomes, liposome diameters 42–175 μm , and (B) schematic diagram for hydrogel formation of chitosan/Di- β -CD matrix [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2.

(A) FTIR spectra of chitosan, Di-β-CD cross-linked chitosan, and its reduced form and (B) schematic diagram for RA-liposome-loaded chitosan-Di-β-CD hydrogels

FIGURE 3.

(A) SEM images of (i) and (iii) chitosan hydrogel and (ii) and (iv) RA-liposomal chitosan hydrogel with chitosan concentrations 3% *w/v* and 2% *w/v*. Di-β-CD (1:0.07 molar ratio). (B) Storage modulus of chitosan hydrogel and RA-liposomal chitosan hydrogel as a function of different chitosan concentrations (3%, 2%, and 1% *w/v*) and different molar ratios of Di-β-CD (1:0.07, 1:0.04, and 1:0.014). Experiments were carried out using an amplitude sweep from 0.001% to 10% strain, gap width 700 μm, and spindle diameter 12 mm, at 25°C

FIGURE 4.

Cytotoxicity of different components of the formulation. Extracts, obtained from (A) chitosan and (B) lecithin powder as well as ricinoleic acid, were analyzed using MTT assay. All samples are expressed as mean and $\pm$ SD from 4 replicates ($n = 4$). (C) Cytotoxicity of hydrogel extracts (100%) and their dilutions (50%, 10%, 1%) obtained from pure chitosan hydrogels and RA-liposome-loaded chitosan hydrogels are compared. Dotted line represents the viability of control cells. (D) Morphology of 3T3 cells after treatment with sample extracts: A—control, B—chitosan, C—lecithin, D—ricinoleic acid, E—chitosan hydrogel, and F—chitosan-RA hydrogels [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5.

Swelling degree (%) of (A) 3% chitosan hydrogel with 1-hour swelling, (B) 3% chitosan hydrogel 24 hours, (C) RA-liposomal chitosan hydrogel 1-hour swelling, (D) RA-liposomal chitosan hydrogel with varying chitosan concentrations and different molar ratios of Di-β-CD, and (E) release profile of ricinoleic acid encapsulated into liposomal chitosan (3% w/v) matrices with and without PA-β-CD at pH 7.4

FIGURE 6.

Wound areas (cm²) were treated with different formulation (A): (1) no treatment, (2) RA-free hydrogel, (3) commercial ream, and (4) test formulation RA-liposomal hydrogel. Chitosan used in 3% w/v cross-linked with PA-B-CD 1:0.04 molar ratio. (B) The (i) 2-cm² wound area before treatment and (ii) 0.04-cm² wound area following 21-day treatment test formulation. (C) Corresponding H & E stains for the harvest tissue for 4 different groups [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1

The gelation rate of chitosan hydrogel as a function of chitosan and Di- β -CD concentrations at different temperatures

Gelation Rate in Minutes										
Gelation Temp. (°C)	Chitosan 3% and Di- β -CD Molar Ratios			Chitosan 2% and Di- β -CD Molar Ratios			Chitosan 1% and Di- β -CD Molar Ratios			
	1:0.07	1:0.04	1:0.014	1:0.07	1:0.04	1:0.014	1:0.07	1:0.04	1:0.014	1:0.014
60	20	30	50	60	75	90	NG	NG	NG	NG
37	30	50	60	80	90	170	NG	NG	NG	NG
25	40	120	130	150	170	NG	NG	NG	NG	NG

^aNG, no gelation formed.

TABLE 2

Histopathological investigation of the treated samples

Histopathological Alternation	Group No.			
	1	2	3	4
Focal acanthosis in the prickle cell layer of the epidermis	-	++	+++	-
Granulation tissue formation in the dermis	-	+++	++	-
Inflammatory cells infiltration in the dermis	-	++	-	-
Inflammatory cells infiltration in subcutaneous tissue	-	+++	+	-

+++ Severe
++ moderate
+ mild
- nil.