CHITOSAN GALLIC ACID MICROSPHERE INCORPORATED COLLAGEN MATRIX FOR CHRONIC WOUNDS: BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION

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ABSTRACT

Objective: Extracellular matrix(ECM) mimicking biomaterials are more effective in chronic wounds than the topically applied healing agents. An attempt has been made to design a unique collagen biomaterial incorporated with chitosan gallic acid microsphere (CGMC).

Methods: Designed CGMC matrix were subjected to various biophysical and biochemical analysis like FTIR, DSC, Drug release analysis, cell toxicity, antioxidant, antimicrobial and in silico molecular modeling.

Results: FTIR studies revealed that incorporation of microsphere to the matrix does not alter the triple helicity of collagen matrix and it is supported by modeling studies. Drug release study also showed that CGMC was able to release the Gallic acid in a sustained manner up to three days at varied pH's (7.4 and 5.4). From the biochemical studies, it was inferred that CGMC does not possess toxicity; it shows high antioxidant potential and also acts as an antimicrobial agent.

Conclusion: our research analysis, through various physical and biological characterization it confirms that CGMC biomaterial has promising bio-properties which are crucial for wound healing and it can be further used for the healing applications.

Keywords: Chitosan, Gallic acid, Collagen, Biomaterials, Microsphere, Modeling, Cytotoxicity.

INTRODUCTION

The microenvironment of chronic wound is a complicated process of un-orchestrated molecular events with improper angiogenesis, ECM deposition and cell migration [1]. In chronic conditions, patients need an intricate robustic biomaterial which mimics the mechanical aspects and sequential assembly of natural tissue [2]. Wound dressings should preserve humid environment, prevent infections, absorb wound exudates, support cell growth, biocompatible, nontoxic and user friendly [3,4].

In spite of the textural assembly of biomaterial, formulation of drug and release pattern remains great importance. A possible method of prolonged release of drug is by entrapment it in a polymer matrix [5]. Among the polymers, chitosan has been extensively used for the development of drug carriers [6, 7]. Though chitosan shows much higher mechanical strength, it has low bioactivity and high brittleness [8]. Among recently available dressings, collagen based products have found to be more effective. They accelerate wound healing as their scaffolds possess high biocompatibility, provides moisture and also capable to regulate the drug release profiles of incorporated drugs [9, 10]. The tripeptide RGD (Arg-Gly-Asp) group in collagen actively associated, like poor mechanical strength and fast degradation. This interaction plays an important role in cell growth, differentiation and overall regulation of cell functions [11]. Even though collagen based dressings were found to be beneficial, there are certain demerits associated, like poor mechanical strength and fast degradation. Upon appropriate integration of both, Combination of chitosan with collagen might lead to unique hardness and brittleness because of increase in mechanical strength. In order to avoid this, chitosan can be formulated as microsphere with the drug which in turn can be incorporated into collagen for the triple combination stable matrix.

Chronic wounds need orchestrated way of drug therapy, which controls the inflammation, minimize the infection in wound environment and also enhances the proper wound closure [12-15]. Gallic acid is a potent antioxidant which has varied activities like anti-inflammatory, broad antimicrobial and angiogenesis [16].

The objective of this study was to develop a Chitosan-Gallic acid microsphere integrated Collagen matrix. Stability and drug release property of matrix was evaluated using various biophysical and biochemical parameters.

MATERIALS AND METHODS

Preparation of chitosan - Gallic acid Microsphere (CGM)

Chitosan-Gallic acid microsphere was prepared by emulsification phase separation method [17]. Gallic acid and chitosan were dispersed together to form a viscous solution by using acetic acid. The solution was added drop wise to the liquid paraffin containing span80 and stirred. After the W/O emulsion, the crosslinking agent glutardaldehyde was added. The cross linked microspheres were separated by filtration, washed and dried at room temperature.

Preparation of Chitosan - Gallic acid Microsphere incorporated collagen matrix (CGMC)

Collagen was isolated from Bovine Achilles tendon by the standard procedure [18]. Isolated collagen was lyophilized and freeze dried. Known amount of CGMC was dispersed in 100 ml of diluted acetic acid. The dispersed solution was poured in the teflon trays and allowed to air dry for 1-3 days.

FT-IR analysis

Functional group analysis for chitosan, CGM and CGMC were determined by collecting FT-IR spectrum (PerkinElmer Instruments, Branford, CT, USA). All spectra were recorded with the resolution of 4 cm$^{-1}$ in the range of 400 to 4,000 cm$^{-1}$ with 20 scans.

DSC analysis

DSC analysis for CGMC and native collagen were analyzed using a differential scanning calorimeter (model DSC Q 200, TA
In vitro drug release from Chitosan-Gallic acid Microsphere (CGM)

Briefly, 25 mg of CGM was placed in microfuge tubes with 100 mM phosphate buffer (pH 7.4 and 5.4) and gently shaken. At regular intervals, supernatants were periodically removed and replaced with equivalent volume of fresh respective buffer. The amount of Gallic acid released at regular intervals was measured using UV spectrophotometer at 234 nm. The average value was calculated from triplicate assays.

In vitro Hemolytic assay

Haemolytic effect of CGMC was evaluated by using human erythrocytes. For the preparation of human erythrocytes the methods of Malagoli was followed [20]. 500 µl of human erythrocytes solution was added to CGMC. Erythrocytes with 0.1% triton x100 acts as a positive control (100% haemolysis) and erythrocytes with 0.85% saline acts as a negative control (0% haemolysis). After 30 min of incubation at room temperature, samples were centrifuged and the supernatant was used to measure the absorbance of liberated haemoglobin at 540 nm. The average value was calculated from triplicate assays.

In vitro cytotoxic assay

Heparinised blood obtained from normal healthy volunteers was layered over lymphoprep gradient (Sigma chemicals) centrifuged at 1880 rpm for 40 min and top two thirds of the supernatant was removed. Peripheral blood lymphocytes (PBL) were aspirated and washed twice with DMEM-F12 medium (Sigma. USA) [21]. Lymphocytes cells diluted in DMEM medium. About 1 x 10^5 cells were loaded in 96 wells. Cells were treated with CGMC and gallic acid and incubated for 24 hrs. At the end of the incubation, 10 µl of 12 mM MTT (3-(4,5-dimethyl thiazolyl)-2-yl)-2.5-diphenyltetrazolium) was added to 200 µl of cells and medium in a 96-well plate. The cells were incubated with MTT for 4 hr. Then, 100 µl of a 5:2:3 N,N-dimethylforamide (DMF): sodium dodecyl sulfate (SDS): water (pH 4.7) was added to dissolve the formed formazan crystals. The results were recorded by using a microplate reader at 585nm.

Antioxidant assay

Lymphocytes were isolated as explained in previous assay [21]. Cells were diluted about 1 x 10^5 and loaded in to 96 well plates. Cells were treated with 5 µl of Fenton’s reagent and incubated with CGMC and gallic acid (GA), respectively. Cells without the Fenton’s reagent acts as positive control. Cells without CGMC and GA with Fenton’s reagent acts as negative control. After 3 h of incubation, cells were treated with 10 µl of MTT and incubated for another 2 h. The formazan crystals formed were dissolved in 5:2:3 ratio of DMF: SDS: H₂O (pH 4.7). The colour developed was analyzed using a microplate reader at 585 nm.

FTIR Studies - CGM

FTIR study was conducted to monitor the chemical modifications in chitosan upon interaction with gallic acid. Characteristic peaks of chitosan (Fig. 1) in the region 3424, 1564, 1414 cm⁻¹ were observed in CGM (Fig. 2) without any significant changes. This suggests that there were no changes in the main backbone of the chitosan structure.
Fig. 1: FTIR Spectra of Chitosan Microsphere

Fig. 2: FTIR Spectra of Chitosan Gallic acid Microsphere (CGM)

Fig. 3: Release profile of Gallic acid from CGM at pH 7.4. Each value is expressed in a mean ± SD (n = 3)

Fig. 4: Release profile of Gallic acid from CGM at pH 5.4. Each value is expressed as a mean ± SD (n = 3)
Fig. 5: FTIR spectra of Chitosan Gallic acid Microsphere incorporated Collagen Matrix (CGMC)

Fig. 6: DSC spectra of Collagen Matrix

Fig. 7: DSC Spectra of Chitosan Gallic acid Micropshere incorporated Collagen Matrix (CGMC)
Fig. 8: The Collagen:Chitosan complex shows the binding of chitosan to the collagen. The collagen is shown in surface view model with triple helices coloured in green, orange and blue. Chitosan is depicted as spheres.

DSC studies CGMC

DSC studies were performed to understand the thermal behaviour of CGMC in comparison with native collagen. From the results, we observed that the interaction of chitosan gallic acid microsphere with collagen increases the thermal stability of CGMC (Fig. 6) than native collagen (Fig. 7). This was inferred from the variation in transition temperature of CGMC (82.9 °C) than native collagen (79.9 °C). Change in the transition temperature of CGMC in turn suggests that the incorporation of CGM to Collagen increases the strength of the biomaterial.

Docking Studies

Molecular docking study of chitosan and collagen was undertaken in order to examine whether the binding of chitosan induces any conformational changes in the collagen structure. Chitosan interacts with collagen through several hydrogen bonds between both partners. Surface view model of collagen:chitosan complex is shown in (Fig. 8).

Chitosan interacts with collagen through hydrogen bonding and hydrophobic interactions. Several amino acids and water molecules of collagen favours in hydrogen bond formation with chitosan (Figure. 9).

Fig. 9: Hydrogen bonding and hydrophobic interactions between chitosan and collagen are shown. Amino acids and water molecules of collagen involved in hydrogen bonding are shown as solid arrow lines

Hydrogen bonding interaction between chitosan and collagen might improve the mechanical property of biomaterial [32] which was further supported by DSC analysis that shows the altered transition temperature of CGMC in comparison with native collagen. Superposition of native collagen and collagen:chitosan complex (Figure. 10) was carried out using Protein3DFit and the root mean square deviation between the main chain atoms was calculated as 0.169 Å. This superposition of native collagen and collagen:chitosan clearly predicts that hydrogen bond formation between chitosan microsphere and collagen does not alter the triple helicity of collagen, as expected. Also, FTIR analysis revealed that the incorporation of chitosan does not alter the triple helicity of the collagen matrix.

Fig. 10: Superposition of native collagen (PDB id: 1BKV, blue) and Collagen: Chitosan complex (shown in orange) reveals no structural change. Chitosan is represented in stick model

Fig. 11: Release profile of Gallic acid from CGMC at pH 5.4 and 7.4. Each value is expressed as a mean ± SD (n = 3)

Fig. 12: In vitro hemolytic activity of Microsphere on human erythrocytes. Each value is expressed as a mean ± SD (n = 3). CGMC, GA, T100 represents Collagen Chitosan Gallic acid Micropshere incorporated collagen matrix, Gallic acid, Triton x100, respectively

In vitro release study

The cumulative percentages of gallic acid released over time at pHs 5.4 and 7.4 were presented in Figure 11. There was not much significant change observed in release profile of gallic acid in CGMC group in varied pHs when compared to CGM. About 0.25% of drug was released within 3 h and the cumulative release gradually increased to 95% at the end of the third day. The pH in the wound is related to the tissue type and not on the grade of the wound. So it is important that the healing matrix need to be stable in variation of pH as healing progresses [29]. Figure 10 clearly suggests that CGMC maintains the drug stability and releases it in a sustained manner at both pHs 5.4 and 7.4.

Toxicity assay

In the present study, we also intend to rule out the possible cytotoxic mechanism of CGMC. Results of in vitro cytotoxicity test for CGMC were given in Figure 12.
confirmed that CGMC biomaterial has promising bio-properties. Incorporation of collagen matrix were investigated. The FTIR study showed the non toxic nature of CGMC, thus making it appropriate for wound healing application.

**CONCLUSION**

MTT reduction showed that CGMC effects on lymphocytes were similar to control groups. Hemolytic assay (Figure 11) reveals that the exposure of CGMC to RBC does not induce any lysis. This clearly shows the non toxic nature of CGMC, thus making it appropriate for wound healing application.

Antimicrobial activity

Microbial infection delays the healing of the wounds. Apart from the biocompatible and biodegradability, the biomaterial should also minimize the wound infection. Microbial load in the wounds hinder the normal healing process and makes it more complicated. In this condition, biomaterial should promote healing by reducing microbial infection. From the antibacterial assay, we found that zone of inhibition produced by CGMC was 22 mm and for ciprofloxacin it was 24 mm. This clearly suggests that CGMC was able to control the infection.

**REFERENCES**


