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Original Article

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, non-toxic 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 entrapping 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 induces cellular adhesion by binding to integrin receptors and 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. Aforementioned demerits may be one of the reasons for the lack of collagen ability to possess prolonged release of drugs.

The problems associated with collagen and chitosan can be effectively balanced 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 formed 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 glutaraldehyde 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 Achillles* 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

Instruments) with standard mode at nitrogen (50 ml min^{-1}) atmosphere with ramp at 10 °C min^{-1}.

***In vitro* drug release from Chitosan-Gallic acid Microsphere (CGM)**

Briefly, 25mg of CGM was placed in microfuge tubes with 100 mM phosphate buffer (pH 5.4 and 7.4) and gently shaked. 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 spectrophotomer at 234 nm. The average value was calculated from triplicate assays.

***In vitro* drug release from Chitosan Microsphere incorporated Collagen matrix (CGMC)**

Gallic acid release from CGMC was analyzed using the Franz-type diffusion cells at 37 °C [19]. The receiver compartment was filled with phosphate buffer solution (PBS pH 7.4 and 5.4), which was stirred with a magnetic stirring bar. At regular time intervals, 1 ml of buffer was removed from the receiver compartment and replaced with an equal quantity of respective buffer to maintain a constant volume. The amount of gallic acid released in the supernatant was determined by measuring the absorbance at 234 nm using UV spectrophotometer. 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 10^6 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-dimethylthiazol-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 10^6 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 act as positive control. Cells without CGMC and GA with Fenton's reagent act 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: H_2O (pH 4.7). The colour developed was analyzed using a microplate reader at 585 nm.

Antibacterial Assay

The antibacterial activity of the CGMC was tested by the disc diffusion method [22]. The test microorganism was seeded into respective solidified agar plate by spread plate method. After spreading, 5 mm CGMC matrix was placed on agar plate along with positive control ciproflaxicin disc and filter paper impregnanted with 100 µg of Gallic acid. The antibacterial assay plates were incubated at 37 °C for 24 h. The diameter of the inhibition zones were measured in mm.

Molecular Docking and binding energy calculations

Chemical structures of Chitosan and gallic acid were generated using ACD/Chemsketch Freeware [23]. The three-dimensional structure of collagen was retrieved from Protein Data Bank (PDB id: 1BKV) [24]. Molecular modeling and docking studies were performed using molecular modeling software, Schrodinger-Maestro [25]. The coordinates of collagen, chitosan and gallic acid were subjected to Protein and ligand preparation wizards, respectively, followed by energy minimization. Ligprep (ligand preparation wizard) assigns or performs addition of hydrogens, 2D to 3D conversion, realistic bond lengths and bond angles, low energy conformation with correct chiralities, ionization states, tautomers, stereo chemistry and ring conformation. Energy minimization of chitosan and gallic acid were performed using OPLS_20005 force field with 1000 cycles of steepest descent and 5000 cycles of conjugate gradient. Addition of hydrogen, correct bond order assignment, charge fixing, amino acid flips were optimized for collagen structure using protein preparation wizard and subjected to energy minimization. Energy minimized models were further used for molecular docking procedure to explore Collagen-Chitosan and Chitosan-gallic acid interactions. Schrodinger-Maestro, PyMOL [26] and Chimera [27] softwares were used for graphical visualization, analyzing hydrogen bond interactions and producing quality images.

RESULTS AND DISCUSSION

Although chronic wounds are common, treatment for such wounds remains limited and largely ineffective. Recently, scaffolds comprised of natural materials were developed for the treatment for the chronic wound and found to be very promising [28]. However, many scaffolds rely on modifications in order to comply with the necessary requirements for current therapeutic needs. Here, we report the preparation and characterization of novel biocompatible biomaterial – collagen incorporated with Gallic acid loaded chitosan microsphere.

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.

***In vitro* Release Study**

In vitro release studies were carried out in both acidic and physiological *pH* to stimulate the *in vivo* conditions in wound area. pH environment in chronic wounds will be slightly basic. As the healing progresses, the *pH* moves towards acidic [29]. The drug release profile obtained for CGM at pH 7.4 (Fig. 3) reveals that it was able to release 95% of drug in sustained manner. This indicates that CGM will be ideal in delivering drug at *pH* 7.4.

CGM release studies at *pH* 5.4 (Fig. 4) shows that it releases 98% of the drug within two hours. This clearly determines the high dissolution and instability nature of CGM at acidic *pH*. The acid instability of the microsphere can be explained by increased proton concentration in acidic *pH* which shifts equilibrium towards solubilization of CGM [30]. In order to overcome the instability nature of CGM at acidic *pH*, it was incorporated into collagen matrix.

FTIR studies CGMC

FTIR studies of the CGMC (Fig. 5) shows the characteristic bands of the collagen molecule and no additional bands were observed. Triple helix integrity is the main feature for the collagen to maintain its biological and mechanical properties. Collagen triple helix integrity can be evaluated by the ratio between the absorbance at 1235 and at 1450 cm^{-1} and the ratio of the denatured collagen will be around 0.5 [31]. The value obtained for CGMC is 1.1 which indicates that the incorporation microsphere does not denature the integrity of collagen.

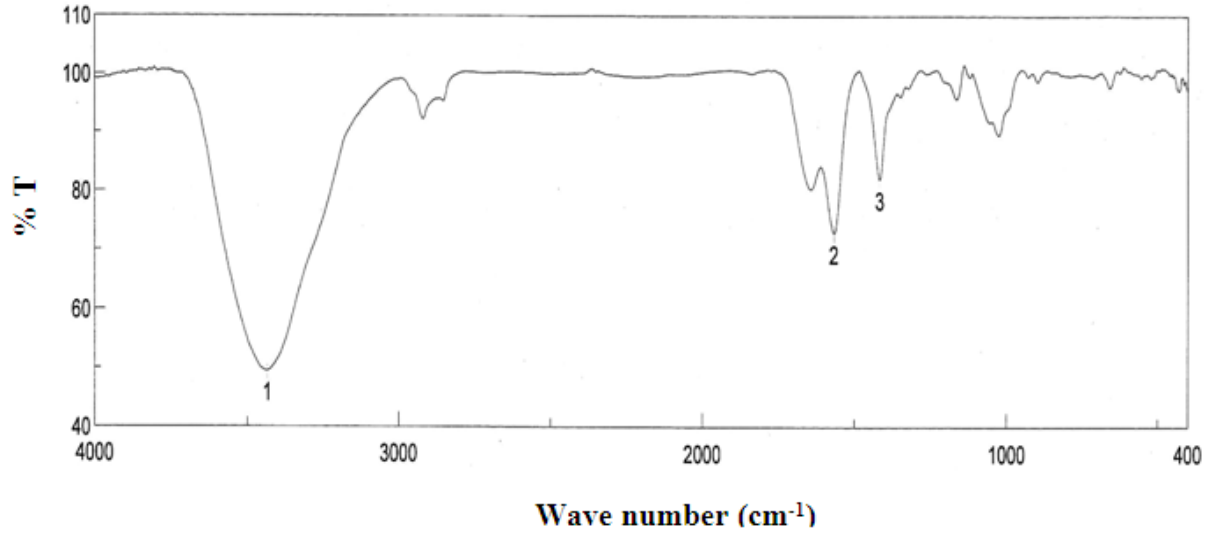

Fig. 1: FTIR Spectra of Chitosan Microsphere

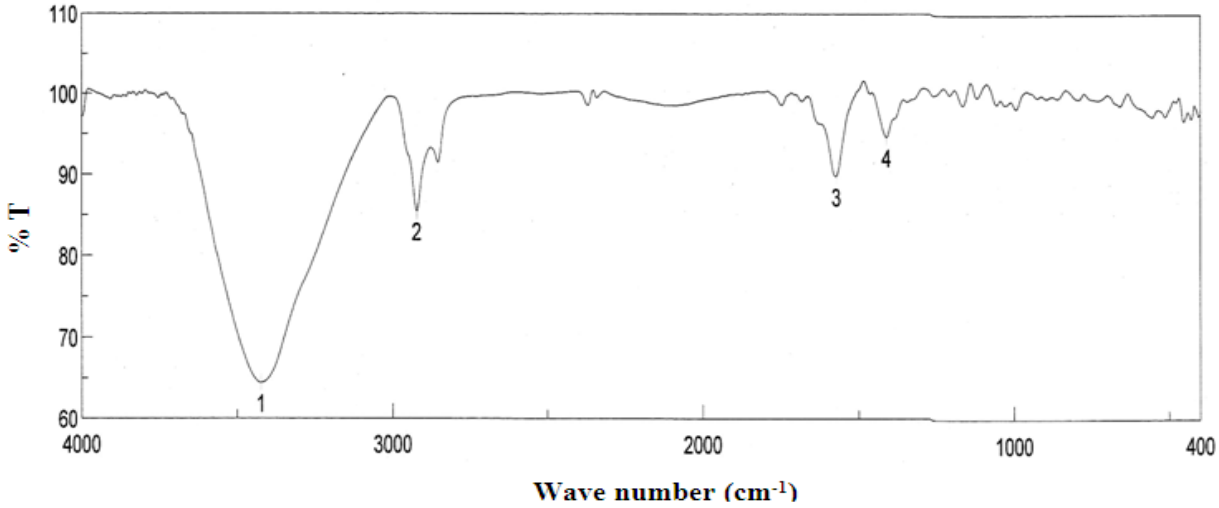

Fig. 2: FTIR Spectra of Chitosan Gallic acid Microsphere (CGM)

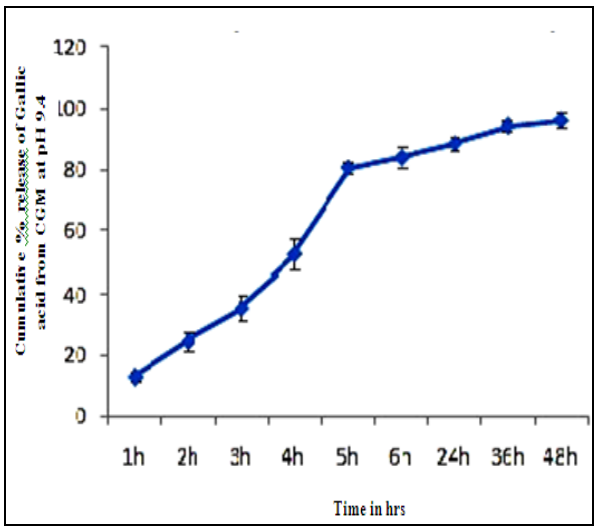

Fig. 3: Release profile of Gallic acid from CGM at *pH* 7.4. Each value is expressed in a mean ± SD (n = 3)

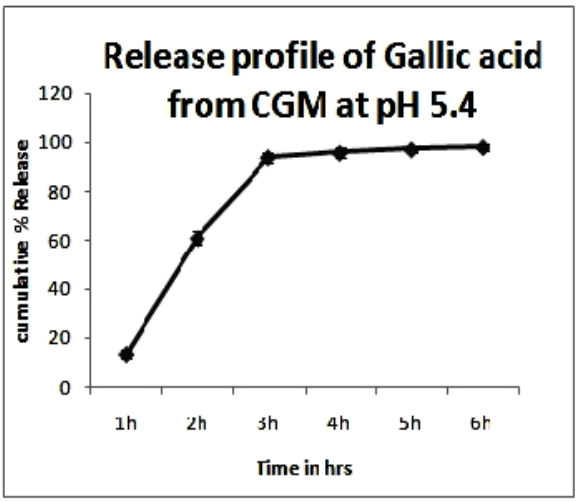

Fig. 4: Release profile of Gallic acid from CGM at pH 5.4. Each value is expressed as a mean ± SD (n = 3)

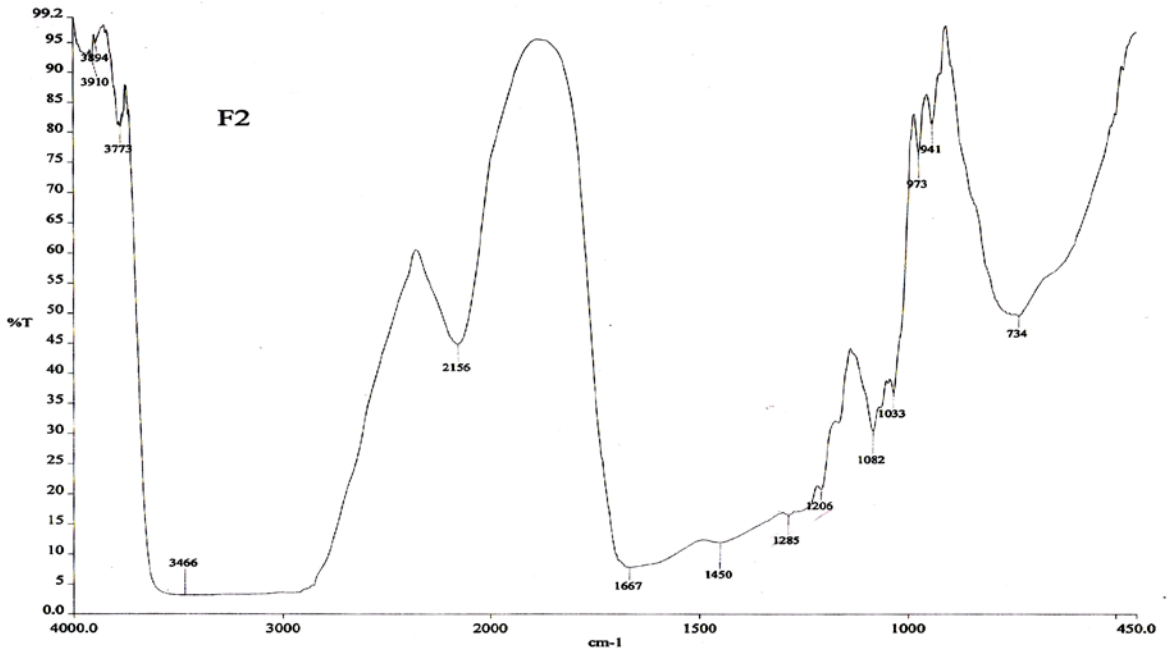

Fig. 5: FTIR spectra of Chitosan Gallic acid Microsphere incorporated Collagen Matrix (CGMC)

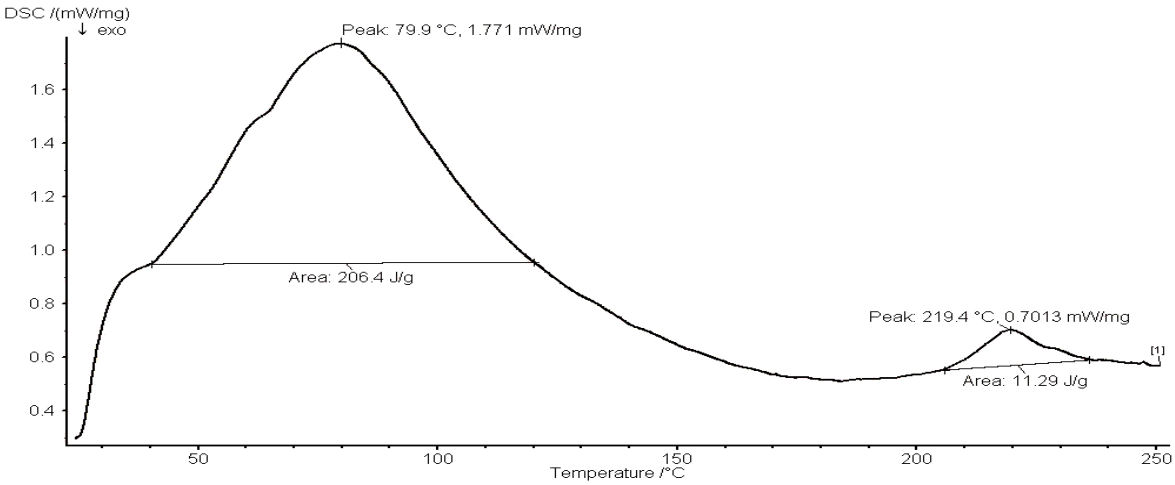

Fig. 6: DSC spectra of Collagen Matrix

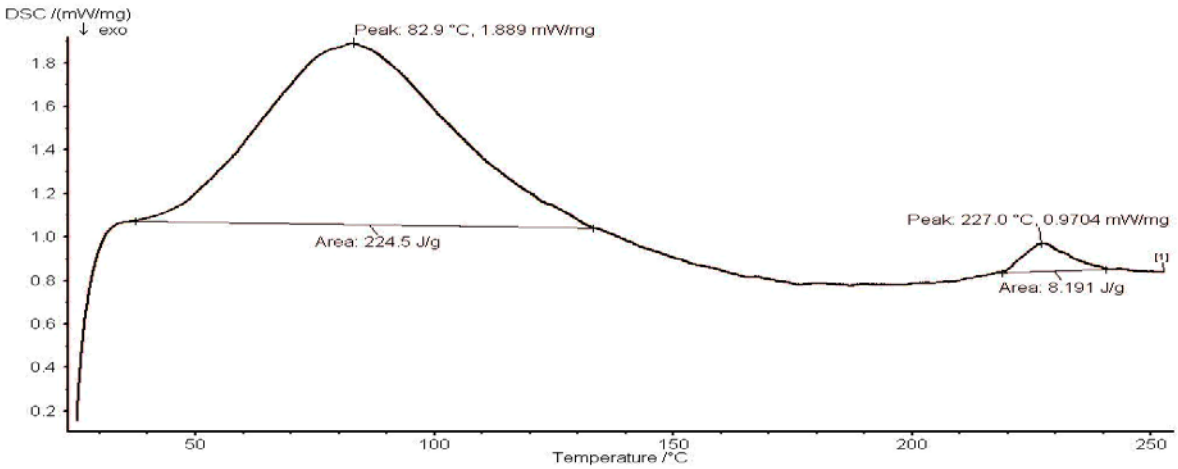

Fig. 7: DSC Spectra of Chitosan Gallic acid Micropshere incorporated Collagen Matrix (CGMC)

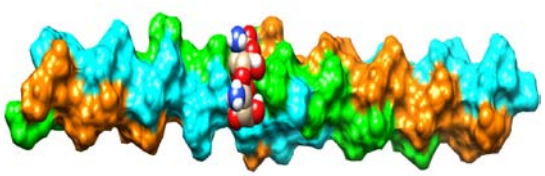

Fig. 8: The Collagen:Chitosan complex shows the binding of chitosan to the collagen. The collagen is shown in surface view model with triple helices coloured in green, orange and blue. Chitosan is depicted as spheres.

DSC studies CGMC

DSC studies were performed to understand the thermal behaviour of CGMC in comparision 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).

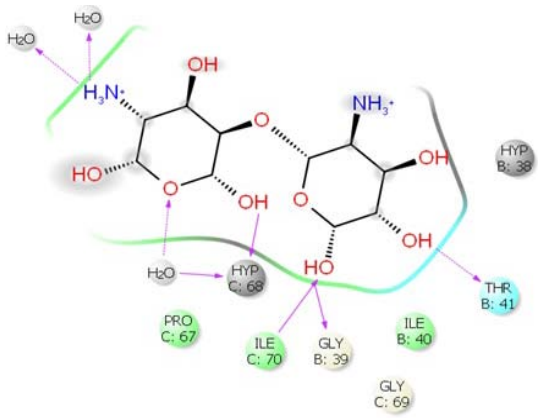

Fig. 9: Hydrogen bonding and hydrophobic interactions between chitosan and collagen are shown. Amino acids and water molecules of collagen involved in hydrogen bonding are shown as solid arrow lines

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 comparision 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 superimposition 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.

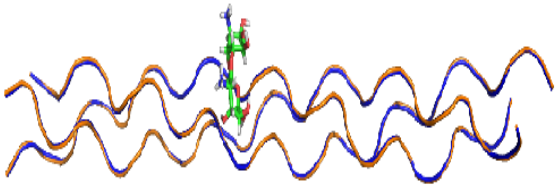

Fig. 10: Superposition of native collagen (PDB id: 1BKV, blue) and Collagen: Chitosan complex (shown in orange) reveals no structural change. Chitosan is represented in stick model

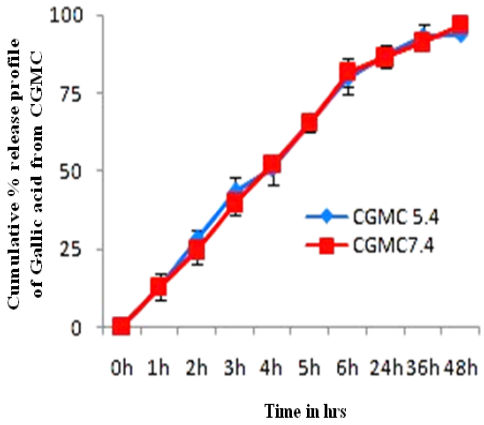

Fig. 11: Release profile of Gallic acid from CGMC at pH 5.4 and 7.4. Each value is expressed as a mean ± SD (n = 3)

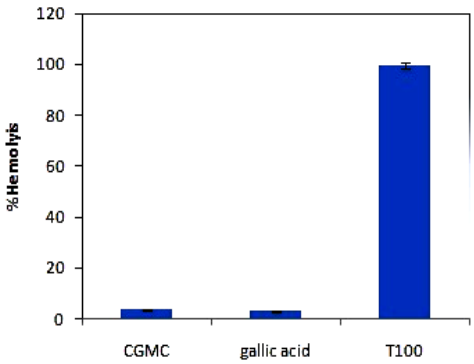

Fig. 12: *In vitro* hemolytic activity of Microsphere on human erythrocytes. Each value is expressed as a mean ± SD (n = 3). CGMC, GA, T100 represents Collagen Chitosan Gallic acid Micropshere incorporated collagen matrix, Gallic acid, Triton x100, respectively

In vitro release study

The cumulative percentages of gallic acid released over time at *pH*'s 5.4 and 7.4 were presented in Figure11. There was not much significant change observed in release profile of gallic acid in CGMC group in varied *pH*'s 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 *pH*'s 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.

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.

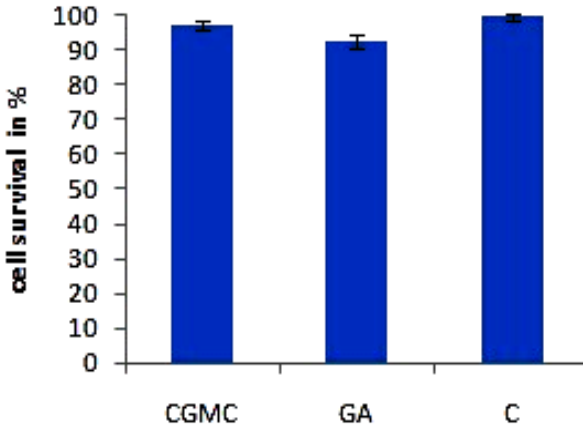

Fig. 13*: In vitro* toxicity analysis of Microsphere on Lymphocytes. Each value is expressed as a mean ± SD (n = 3). CGMC, GA, C represents Collagen Chitosan Gallic acid Micropshere incorporated collagen matrix, Gallic acid, control, respectively

Antioxidant activity

Antioxidants counter the excess proteases and reactive oxygen species (ROS) often formed by neutrophil accumulation in the wounded area and protect protease inhibitors from oxidative damage. Fibroblasts and other cells may be killed by excess ROS and skin lipids will be made less flexible, so antioxidant substances will reduce the possibility of the occurrence of these adverse events. Because of these several factors, overall antioxidant effects appear to be important in the successful treatment of wounds [33]. From the antioxidant study (Figure 14), it was inferred that on treatment of free radical to lymphocytes in the presence of CGMC, the cell survival was found to be 80%. This clearly suggests that CGMC was able to scavenge the radicals and extend the survival of lymphocytes. On the other hand, cells treated with the same amount of gallic acid alone which was present in the CGMC showed 94% of cell survival. This difference in cell survival in CGMC and gallic acid may be due to sustained release of gallic acid from CGMC which causes variation in concentration of gallic acid. This also suggests that slow release of gallic acid from CGMC prevents the gallic acid overload at the environment and helps to scavenge the free radicals which are generated in regular intervals at wound environment.

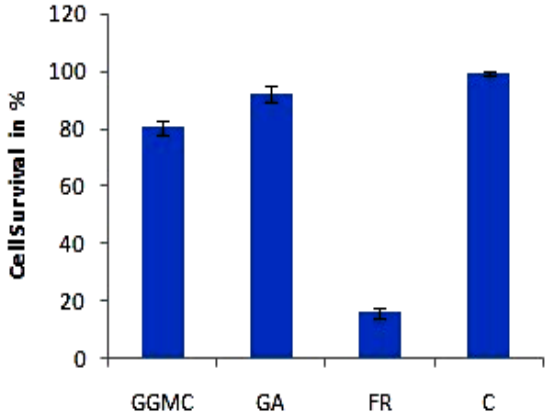

Fig. 14: *In vitro* antioxidant activity of Microsphere. Each value is expressed as a mean ± SD (n = 3). CGMC, GA, FR and C represents Collagen Chitosan Gallic acid Micropshere incorporated collagen matrix, Gallic acid, Fentons Reagent and Control, respectively

Table 1: Antibacterial effects of Microsphere on *E.coli* strain (Zone of inhibition in mm)

S. No.	Test Agents	Zone of inhibition (mm)
1	CGMC	20 ± 1
2	Gallic Acid	22.5 ± 1.5
3	Ciprofloxacin	24.66 ± 0.5

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.

CONCLUSION

Biophysical and biochemical properties of Chitosan-Gallic acid incorporated collagen matrix were investigated. The FTIR study clearly shows that incorporation of microsphere into the collagen doesn't alter the structural property and integrity of collagen, which is evident from the molecular docking studies of chitosan and gallic acid. The drug release confirmed that CGMC releases the drug in a sustained manner. Toxicity studies revealed that CGMC doesn't produce toxicity to the cells. From our research analysis, it was confirmed that CGMC biomaterial has promising bio-properties which are crucial for wound healing and it can be further used for the healing applications.

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