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Fabrication, Characterization of Curcumin Loaded Alginate Chitosan for Potential Wound Healing Applications

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Abstract

The most employed scaffolds in tissue engineering are alginate and chitosan due to their properties like biodegradability, compatibility, and structural similarity to that of the ECM. Curcumin, together with scaffolds such as alginate and chitosan, can improve wound healing properties by tissue repair and regeneration. This study aims to load curcumin into alginate chitosan scaffolds and to analyze their potential wound healing properties by characterization and checking their biocompatibility. Curcumin was loaded into the alginate-chitosan scaffold. It was then characterized using Fourier Transform-InfraRed (FT-IR) spectroscopy and Scanning Electron Microscopy (SEM). Annexin V PI apoptotic assay and Hemolytic assay were done to screen its biocompatibility. FT-IR has strong absorption bands at 3237, 2359, 1597, 1406, 1025, and 947 cm^{-1}. SEM analysis of the curcumin-loaded alginate-chitosan scaffold showed the dispersed curcumin on the surface of the porous scaffold. Our results suggest that the curcumin-loaded alginate-chitosan scaffold possesses greater biocompatibility towards peripheral blood mononuclear cells (PBMC) which was confirmed by Annexin V - PI assay and hemolytic assay. Curcumin loaded onto an alginate-chitosan scaffold is reported to be biocompatible using flow cytometry and hemolytic assay. However a more detailed study must be done before using it for potential wound healing applications.

Keywords: *Biocompatibility, Curcumin, Novel Technique, Tissue Engineering, Wound Healing.*

Introduction

Phytochemicals are secondary metabolites produced by plants as a mechanism of defense against pathogens [1]. The compounds have been a part of traditional medicine for decades. Therefore, they have been explored for their medicinal properties and are now used in pharmaceutical, industrial, and food industry applications as well [2–5]. The phytochemicals of flavonoids, alkaloids, terpenoids, saponins, carotenoids, and other aromatic and organic acids were reported in many studies. Such phytochemicals possess anti-microbial, anti-fungal, anti-carcinogenic, anti-mutagenic, anti-inflammatory, anti-oxidative, and many more properties [6–8].

Curcumin is a polyphenol phytocompound of the plant *Curcuma longa* and has been a part of traditional medicine in India and in various parts of South Asian countries, was widely used for its anti-inflammatory, anti-bacterial and anti-viral properties [9]. Curcumin has been explored for its wound-healing properties for more than a decade. Wound healing is a process of tissue repair and remodelling in response to barrier damage [10]. Curcumin contains two aromatic rings along with methoxyl and hydroxyl groups [11]. A 2017 study revealed the effects of

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topical curcumin on the wound-healing capacity of the nasal mucosa and showed a significant reduction in inflammation and accelerated wound healing [12]. In 2022, an in-vivo study revealed the significant effects of curcumin by accelerating collagen deposition and angiogenesis [13]. Topical application of curcumin has been shown to improve the wound healing capacity of the skin [14]. However, it has a few limitations such as it possesses high toxicity when given at high dosages. Therefore, nanocarriers can be used to deliver the curcumin to modulate its limiting factors [15, 16].

Recent researchers are focusing on cross-linking natural polymers. The most commonly employed scaffold in tissue engineering is the alginate and chitosan due to their properties such as biodegradability, compatibility, and structural similarity to that of the ECM. Chitosan is a polycationic polymer that plays a role in cell adhesion, proliferation, and differentiation [17, 18]. Alginate is a polyanionic polymer that plays an important role in tissue regeneration and vascularization [19]. Crosslinking of polymers is found to provide greater stability, mechanical strength, resistance to hydrolysis, and promote better chemical interactions [8, 20, 21]. Biopolymers such as alginate and chitosan have been used as wound dressing for decades due to their non-toxic nature, anti-microbial properties, and availability [22, 23]. Curcumin is also encapsulated in chitosan and used as a film delivery system to enhance the wound healing properties of curcumin [24].

Moreover, curcumin loaded onto a biopolymer system can prolong the delivery of the drug and it also shows a non-hemolytic nature [10]. Many polymers have been shown to reduce the blood flow and epithelial cell migration to the wound site [25, 26]. Therefore, using natural polymers can overcome this limitation when used along with phytocompound can significantly increase the wound healing capacity. Therefore, in this study we have fabricated a Curcumin-loaded Alginate Chitosan Scaffold to be used as a drug delivery system for better cell signalling, enhancing cell growth and tissue regeneration.

The objective of the present study is to prepare a curcumin-loaded alginate chitosan scaffold, characterize its properties and analyze its biocompatibility to confirm its suitability for potential wound healing applications.

Materials and Methods

Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), antibiotic/antimycotic solution, fetal bovine serum, and Trypan Blue were all procured from Himedia. Propidium iodide and Annexin V were purchased from Sigma Aldrich, India. All other reagents used were of analytical grade. MilliQ water was used throughout the study.

Preparation of Alginate–Chitosan Scaffolds

Curcumin solution was prepared by solubilizing 12 mg of curcumin in 1 mL DMSO solution. Then the curcumin solution is mixed with 5mL of oxidized alginate solution for the fabrication of scaffolds. About 500 µL of alginate solution with curcumin was mixed with 5 % chitosan hydrochloride solution into a 12-well plate and the gel formed was lyophilized and used for further studies.

Characterisation of Curcumin Loaded Alginate Chitosan Scaffolds

Curcumin-loaded Alginate Chitosan Scaffolds were characterized using Fourier-transform infrared spectroscopy (FT-IR) and Scanning Electron Microscopy (SEM). The presence of a functional group in Alg-Chi-Cur was determined by FT-IR (Bruker) at a scan range of 4000 to 500 cm^{-1} with a scanning speed of 4 cm^{-1} in ATR mode. Scanning electron microscopy (SEM) (JEOL JSM –

IT800 SEM, Japan) was used to examine the surface morphology of Alg-Chi-Cur.

In Vitro Drug Release

The *in vitro* release of quercetin from the Alg-Chi-Cur scaffold in PBS (0.1 M, pH 7.4) was estimated using a dialysis membrane of MWCO 3.5 kDa. About 10 mg of the scaffold in 10 mL PBS was taken in the dialysis bag and the bag was immersed in a 100 mL beaker with 50 mL PBS. The beaker was incubated at 37°C in a shaking water bath at 50 rpm. Aliquots of 1 mL were withdrawn from the beaker at different time intervals and replaced with the equivalent amount of fresh PBS. The released curcumin was estimated from UV absorbance of the sample at 431 nm [27].

Biocompatibility of Curcumin Loaded Alginate Chitosan Scaffolds in PBMC

For determining the biocompatibility of curcumin loaded Alginate Chitosan Scaffolds in PBMCs, an Annexin V - PI apoptosis assay was performed. Following the approval of the Institute Human Ethical Committee, blood was collected from healthy donors. 2 ml of blood was added over 2 ml of HiSepTM LSM 1077 medium and centrifuged to isolate PBMCs. The viability of PBMCs was confirmed using Trypan Blue Assay. Equal volume PBMCs were seeded in six-well plates and treated with 100 µg of Alg-Chi-Cur and incubated for 12 h. Untreated cells were used as control and were incubated for 12 h. After incubation, cells were harvested, centrifuged, and the supernatant was discarded and resuspended in a binding buffer. The cells were then stained using Annexin V FITC (5µl) and Propidium Iodide (5µl) and incubated at room temperature for 15 minutes. After incubation, 400 µl of 1X binding buffer was added and acquired (10000 events) using BD FACS Lyric flow cytometer, and the treated cells were observed for apoptosis. The analysis was performed using FAC suite 4.1 software.

Hemolytic Assay

To determine the toxicity of Alg-Chi-Cur, a hemolytic assay was carried out. The amount of hemoglobin released from erythrocytes after treatment with Alg-Chi-Cur determines the toxicity. Protocol for this assay was carried out as previously reported by [28]. Human blood was centrifuged at 1500 xg for 5 minutes and plasma was discarded to collect the erythrocytes (RBCs). Phosphate-buffered saline (PBS) with pH 7.4 was used to wash the isolated RBCs thrice. After washing, the obtained RBCs were diluted to 10 % of their initial concentration using PBS to get erythrocyte suspension. 200 µl of erythrocyte suspension was added to samples of varying concentrations (12.5, 25, 50, 100, and 200 µl/ml) and the sample was made up to 1 ml using PBS. The contents were incubated for 1 h at 37°C and centrifuged at 1500 xg for 5 minutes. After centrifugation, the supernatant was loaded in 96 well plates and the absorbance were measured at 540 nm in an ELISA plate reader. Cells treated with PBS were taken as negative control and the cells treated with deionized water were taken as a positive control. The procedure was repeated thrice. The percentage of hemolysis was calculated using the formula (Bulmus et al., 2003),

$$Hemolysis\ Percentage = \frac{O.D\ of\ the\ sample\ -\ O.D\ of\ Negative\ Control}{O.D\ of\ Positive\ Control\ -\ O.D\ of\ Negative\ Control}\ x\ 100$$

Results

In this current study, Curcumin loaded onto Alginate Chitosan Scaffolds was done and it was characterized by FT-IR and SEM, and its biocompatibility is checked by Annexin V apoptotic assay and hemolytic assay.

Characterization of Curcumin Loaded Alginate Chitosan Scaffolds

FT-IR spectra were recorded between 4000 to 500 cm^{-1}. Figure 1. represents the result of

the synthesized Alg-Chi-Cur. The FT-IR spectrum of Alg-Chi-Cur showed strong absorption bands at 3237, 2359, 1597, 1406, 1025, and 947 cm^{-1}. Characteristic absorbance at 3237 cm^{-1} confirms the Alg-Chi-Cur formation, evidenced by the shift in maximum absorbance compared to the extract.

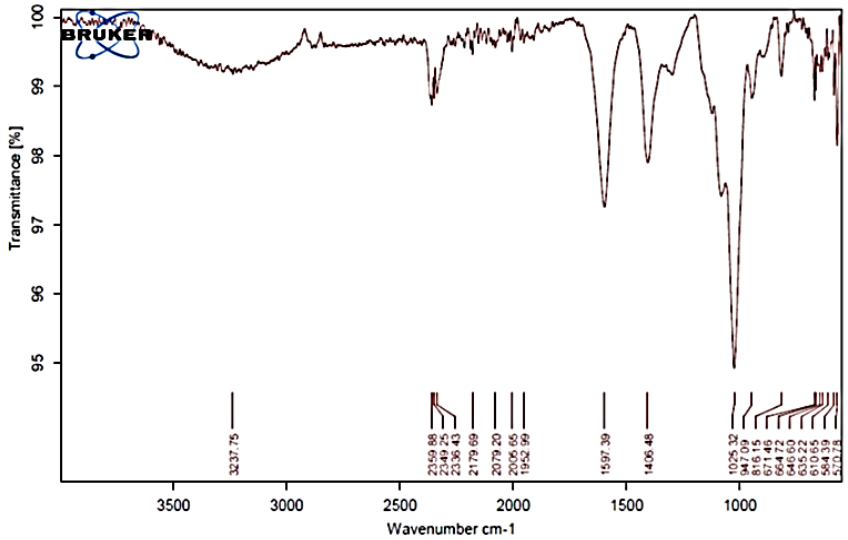

Figure 1. FT-IR Spectrum of Alg-Chi-Cur Scaffolds

The morphology of Alg-Chi-Cur was determined by Scanning Electron Microscopy. The SEM micrograph showed the dispersed curcumin nanoparticles around 100 nm size on the surface of the porous scaffold (Figure 2).

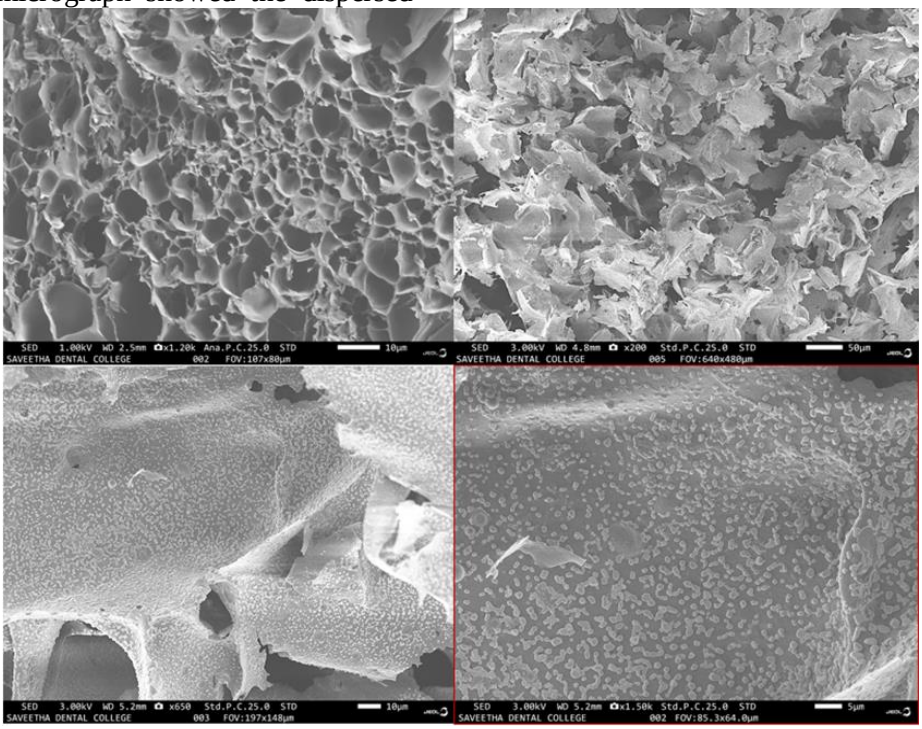

Figure 2. SEM Micrograph of Alg-Chi-Cur

Drug Release Study

The cumulative percentage of quercetin released from the scaffold at pH of 7.4 is given in Figure 3. The results showed a quercetin release of about 16% during 6 h.

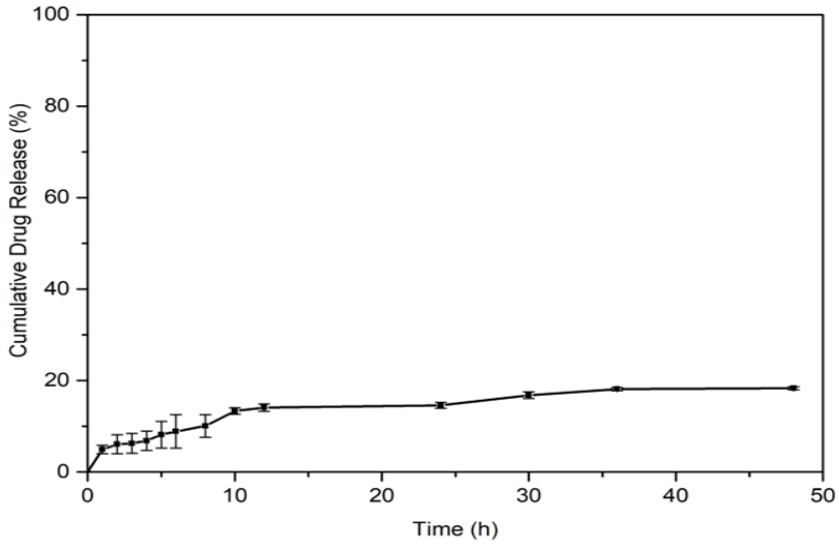

Figure 3. Cumulative Quercetin Release from Alg-Chi-Cur Scaffold

Hemolytic Assay

Alg-Chi-Cur showed less than 5% hemolysis in erythrocytes at various concentrations from 200, 100, 50, 25, and 12.5 µg/mL in comparison with the control. Figures 4 represent the Hemolytic assay test results of Alg-Chi-Cur.

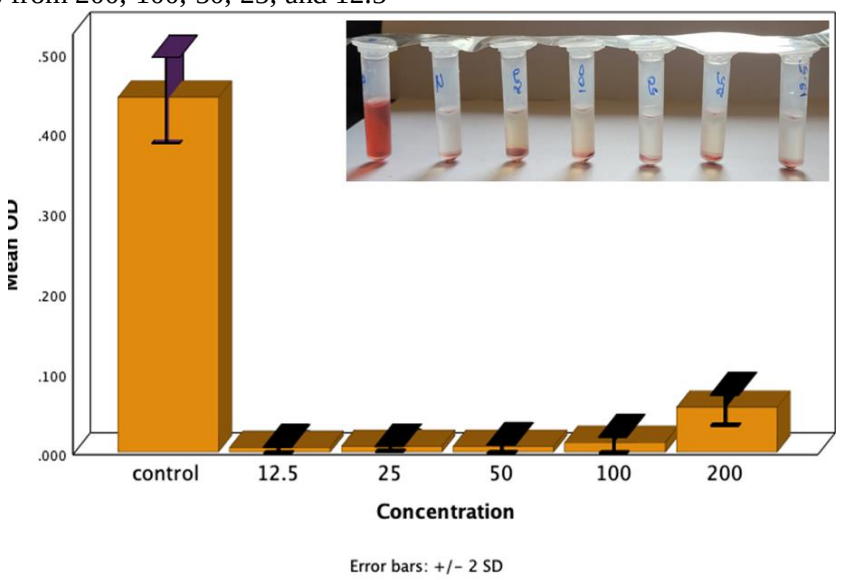

Figure 4. The Visual Image of the Hemolysis Induced by Various Concentrations of Alg-Chi-Cur (200, 100, 50, 25, and 12.5 µg/mL), Negative Control (N), and Positive Control and the Hemolytic Activity of Alg-Chi-Cur.

Figure 5. represents the Annexin V-PI study of PBMC treated with Curcumin Alginate Chitosan Scaffolds. The Annexin V-PI assay results showed the maximum viability of 78.22 % with the Alg-Chi-Cur treated PMBCs, while 21.45 % were found to be in the early apoptotic stage, 0.28 % were found to be in the late apoptotic stage and 0.05 % of the cells showed necrosis. The viability of the cells showed almost significant results when compared with that of the untreated cells. Thus, this biocompatibility study performed using Annexin V and the propidium iodide staining method revealed the non-toxic nature of Alg-Chi-Cur in PBMC.

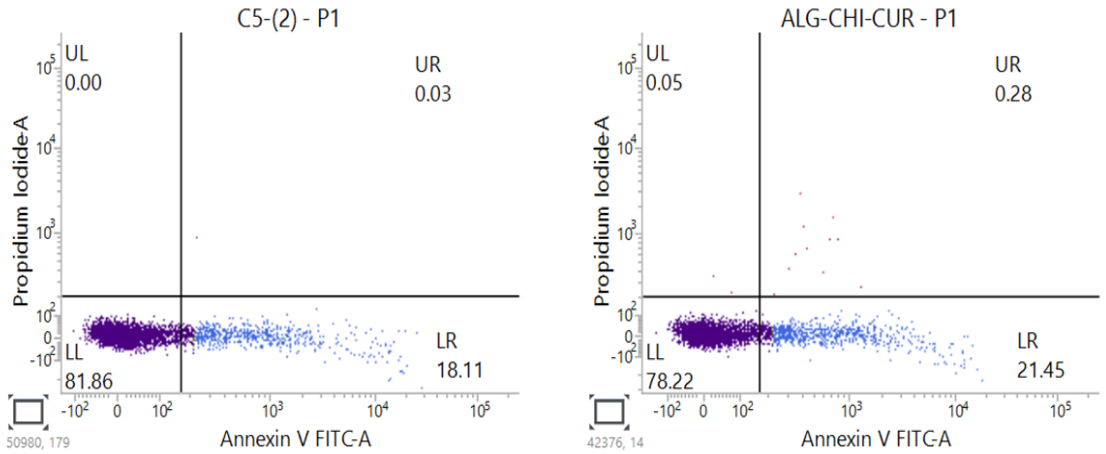

Figure 5. Annexin V and PI assay of a) Control, b) Alg-Chi-Cur

Discussion

Crosslinking of polymers is found to provide greater stability, mechanical strength, resistance to hydrolysis, and promote better chemical interactions [20]. In a study, it is shown that alginate-chitosan film possesses intrinsic antimicrobial activity and improved wound healing properties [29]. Curcumin has been used for centuries for its wide medical applications. It possesses strong antibacterial, and antifungal properties, and a powerful wound-healing capacity owing to its antioxidant activity [30]. Together with scaffolds such as alginate and chitosan, it can improve wound healing properties through tissue repair and regeneration [31].

Considering this, we loaded Curcumin onto an alginate-chitosan and fabricated a scaffold. Curcumin-loaded porous structure was visible in the SEM image [32]. The curcumin loading is confirmed by the FT-IR spectrum [33]. Utilizing flow cytometry, the material's biocompatibility was evaluated and confirmed to be non-toxic in Peripheral blood mononuclear cells [34-38]. FT-IR spectrum results showed strong peaks stretching at 3237, 2359, 1597, 1406, 1025, and 947 cm^{-1} revealing the presence of flavonoids and phenolic compounds in the extract. The peak absorbed at 3237 cm^{-1} shows a strong, broad O-H stretching of carboxylic acid. The peak at 2359 cm^{-1} reveals strong O=C=O stretching of carbon dioxide. The peak at 1406 cm^{-1} shows a medium O-H bending of alcohol. The peak 1025 cm^{-1} shows a strong C-F stretching of the fluoro compound. At the peak, 947 cm^{-1} shows strong C-Cl stretching of the halo compound. The results thus concluded the presence of functional groups such as phenolic group, aromatic group, etc. The SEM results of curcumin-loaded Alginate Chitosan Scaffolds revealed the presence of curcumin nanoparticles on the surface of the porous scaffold. Apoptosis assay revealed that almost 78.22 % of cells were alive after treatment with 100 µg of Alg-Chi-Cur, comparable with that of untreated control showing 81.86 % viable cells; this confirmed the biocompatibility of Alg-Chi-Cur and their non-toxic nature. Hence, curcumin-loaded alginate-chitosan scaffolds could be used for potential wound-healing applications.

Conclusion

Biological scaffolds are used as a drug delivery system for the prolonged delivery of the molecule to the target site. It also offers better solubility and bioavailability of the target compound, and its sustained release of the drug can enhance the wound healing phase. The bioavailability of curcumin is enhanced by loading it onto the alginate-chitosan scaffold. The biocompatibility of the

material was assessed using flow cytometry and found to be non-toxic PBMC. Curcumin is a known powerful antioxidant agent. It also has great scope in the food and medical industries. However, deep in vitro and in vivo testing are essential before using it for various appliances.

We used Curcumin and loaded it onto Alginate Chitosan Scaffolds., characterized their properties using techniques such as FT-IR, and SEM, and toxicity study was evaluated on PBMCs using Annexin V-PI apoptosis assay. The morphology of the Alg-Chi-Cur scaffold examined by SEM showed porous surfaces with dispersed nanosized curcumin of around 100 nm in diameter. Our *in-vitro* study showed that Alg-Chi-Cur does not induce significant apoptosis and showed very little necrosis and hemolysis at 100 µg. Furthermore, the viability of Curcumin-loaded Alginate Chitosan Scaffolds treated PBMCs was similar to that of untreated control. Our findings showed that Alg-Chi-Cur was non-toxic to PBMCs and could be used for biomedical applications. However, further investigation must be carried out to evaluate the *in-vivo* toxicity nature of the Alg-Chi-Cur. Therefore, we concluded that the Curcumin-loaded Alginate Chitosan Scaffolds are economical. Because of its biocompatibility and high mechanical strength as per previous reports, it could be a key component in bone tissue engineering applications.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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