Development Of Glycosaminoglycan Scaffold Integrated With Herbal Nanoparticle For Wound Healing
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Abstract
Glycosaminoglycans, the most abundant heteropolysaccharides, have an inevitable role in tissue hydration, cell adhesion, regulation of cell growth and proliferation. Glycosaminoglycan-chitosan-gelatin (GCG) scaffold incorporated with Hemigraphis colorata silver nanoparticles was prepared. The study confirmed the physical cross linking of the scaffold using scanning electron microscopy (SEM) and showed potent antibacterial and antioxidant activity. GCG scaffold formed almost uniform pore size even without addition of chemical cross linking agent. MTT Cell Proliferation and invitro scratch wound healing assay proved that the said scaffolds are non-cytotoxic, promote cell adhesion and have potent wound healing property. Silver nanoparticles of Hemigraphis colorata with an average size of 23.0nm (as per TEM analysis), integrated in this scaffold enhanced its potential application as burn wound dressing material. GCG scaffold prepared by freezing and lyophilization is soft, spongy and flexible, thus promising its task to advance the burn wound treatment management with release of drug resulting in closure of wound by 48 hours.

Key Words: Glycosaminoglycans, Silver nanoparticles, Hemigraphis colorata, Chitosan, Cytoocompatatability, Scratch wound assay.

I. Introduction
Each year a considerable amount of total fish catch is discarded as processing left overs including trimmings, fins, frames, heads, skin and viscera(1). According to Food and Agriculture Organization of the United Nations, in many countries, solid fish waste is recycled into fish meal or treated along with the municipal waste(2). Treated fish waste has found many applications as animal feed, food-packaging (chitosan) and as moisture maintenance in foods (hydrolysates)(3). The highest concentration of these bioactive compounds is generally found in the discarded parts (4).

Glycosaminoglycans (GAGS) are complex, sulphated polysaccharides including chondroitin sulfate (CS) dermatan sulfate (DS), hyaluronic acid (HA), heparin and heparin sulphate (HS). They are heterogeneous polysaccharides in terms of relative molecular mass and physico-chemical properties, used widely in pharmaceutical, cosmetic and food industries (5). Gelatin obtained through a controlled denaturation of collagen, which is the major component of fish skin, bone, and connective tissue. It is frequently used as matrices for tissue engineering. Gelatin has the advantage of absorbing excess exudates, because of its excellent ability to absorb water up to 5-10 times as its weight (6). The novel properties of chitosan mainly nontoxic, biocompatible and biodegradable polymer with anti-microbial activity can accelerate wound healing by enhancing the functions of inflammatory cells and repairing cells make it a versatile biomaterial for tissue engineering and gene therapy (7).

Natural commodities are a source of traditional herbal medicine. Subramnaiam et al. have first reported the wound healing properties of the leaf of Hemigraphis colorata and have suggested its use in folklore medicine in the treatment of wounds (8). The leaf paste applied on the wound promoted wound healing in mice with much faster wound contraction and epithelialization (9). While exposing
cells or tissues to silver nanoparticles the active surface would be significantly large compared to silver compounds, and there by exhibiting remarkably unusual physiochemical properties and biological activities and show sustained release dose regime (10, 11). The frequency of burn wound sepsis and secondary bacteremia were found to be less in patients treated with silver nanoparticles (12). Nowadays the health care professionals are facing an escalating number of patients in misery from burn wounds, complicated to treat and mend. The usage of biologically active scaffolds for tissue engineering and regenerative medicine is attaining a promising result in wound management and skin replacement. Fortifying the scaffold with bioactive molecules and glycosaminoglycans (GAGs) is an efficient way to design new generation tissue engineered biomaterials (13). The present study focuses on a blend of these natural polymers with incorporated herbal nanoparticles that may perhaps guarantee a well-designed and multipurpose scaffold for burns and wounds.

II. Materials and Methods

Chemicals and materials
Glycosaminoglycan isolated from fish waste (14). Gelatin (type A from porcine skin, 75-100 Bloom) and chitosan from Sigma Chemicals. *Hemigraphis colorata* leaves were collected from in and around of Wayanad district, Kerala. L929 and HaCaT cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecos modified Eagles medium ( Gibco, Invitrogen). The cell lines were cultured in 25 cm² tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany).

2.1. Preparation of *H.colorata* leaf aqueous extract

*H.colorata* leaves were collected washed thoroughly in running tap water followed by distilled water. *H.colorata* aqueous leaf extract was prepared with slight modifications (15).

2.2. Synthesis of silver nanoparticles

Silver nanoparticles were synthesized with 1:4 ratio of plant extract to 1mM AgNO₃ and confirmed by UV- spectrophotometry (16).

2.3. Characterization of AgNPs

The sample analyzed in UV visible spectrophotometer between the wavelengths 200nm to 700nm to obtain the absorption spectra (17). External morphology of the nanoparticles was determined using Transmission electron microscopy (18) at Sophisticated Analytical Instruments facility of the University. Kirby Bauer well diffusion method was adopted to analyze the antibacterial activity as per (19). *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* strains were obtained from CUSAT microbiology laboratory and maintained in nutrient agar slant at 4°C.

2.4. Preparation of silver nanoparticles incorporated GCG scaffold

Scaffolds were made using previously described procedure (20) with minor modifications. In brief, 0.2g chitosan was added to 10ml 0.1% glacial acetic acid and kept in shaking incubator at 37°C and 150 rpm for 12 hrs. The impurities removed by filtering through whatman no. 1 filter paper. Gelatin solution prepared by dissolving 0.2g in 10ml distilled water and heated at 45°C for 30 minutes with continuous stirring. The gelatin and chitosan solutions were mixed 1:1 ratio and to this 0.5g glycosaminoglycan and 1ml *H.colorata* incorporated silver nanoparticles (1%) were added with continuous stirring for complete blending and degassed ultrasonically for 15minutes. The mixture was poured uniformly into petridishes, freezed and lyophilized to obtain the film.
2.5. Characterization of silver nanoparticles incorporated GCG scaffold

2.5.1. SEM analysis of H.col. Ag NPs incorporated GCG scaffold.
The morphology of GCG scaffolds characterized by SEM. Cross-sectional morphologies were viewed using a JSM-6390 operated at VOLT 20 accelerating voltage. Pore size was measured using a submenu, consisting on a ruler tool, incorporated in the software of the SEM equipment. Once the overall cross-sectional images of the scaffolds were obtained, several pores (>20) were selected. In each selected pore, the micron marker 50um measured the diameter of the pore by measuring the distance between two points. In each cross-sectional image of the scaffolds, the average pore size was computed.

2.5.2. Swelling studies
Studies on swelling behavior were done with slight modification of (21). The samples of size 2x2 cm with 4mm thickness were cut and dried in hot air oven equipped with vacuum pump at 37°C for 24hrs, weighed and denoted as WO. Then immersed the samples in distilled water and phosphate buffered saline ( PBS ) pH 7.2 at 37 °C . Took weight after frequent intervals and denoted as W1. Swelling ratio was calculated using the formula (R)=(W1-WO) X 100

2.5.3. Free Radical-scavenging activity –DPPH Method.
0.1g of GCG Scaffold with and without incorporated herbal AgNp were dissolved in 2 ml distilled water at 45°C followed by the addition of ethanol (4 ml) and centrifuged in Remi cooling centrifuge at 6000×g for 10min at 20°C. The filtrate obtained was evaluated for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (22) with slight modifications.

2.5.4. Antimicrobial activity of AgNPs incorporated GCG scaffold.
The antimicrobial activity of AgNPs incorporated GCG scaffold against Staphylococcus sp., Pseudomonas sp., was studied according to Kirby Bauer standard well diffusion method on Muller Hinton Agar (MHA) plates.

2.5.5. Determination of cytotoxicity in Cell culture.
The cytotoxicity of materials extracts was evaluated as per ISO10993-5 on L929 cell culture. Scaffolds sterilized in UV and immersed in DMEM overnight. The medium was extracted and cells were seeded on the surface of scaffold and allowed growing under standard conditions. The percentage of the surviving fibroblast cells were quantified by the MTT assay and the morphological changes of the cells were monitored by phase contrast microscopy (23).

2.5.6. Scratch wound healing assay.
Exponentially growing cells were trypsinized and seeded at a density of 200,000 cells per well into 12-well plate for 24 h incubation (~90% confluence). The scratch wounds were made by a sterile 1 ml pipette tip through a pre-marked line. After removal of the resulting debris from five lineal scratches, the cell monolayer was subsequently rinsed three times with PBS followed by incubation with polymer scaffold for 24 hours. The wound areas were displayed by taking images just above the interchanges between scratched wound areas and pre-marked lines and the effect of polymer on wound closure was determined microscopically (20X magnification, Olympus CKX41) after 24 and 48 hours of incubation. The effect of material on wound closure was measured in terms of area using MRI-Image analysis software.

III. Results

3.1. Physical Characterization of H.col. AgNp:
Formation of H.col. silver nanoparticles were confirmed by the reduction of silver ions which was visibly evident from the color changes associated with it (Fig.1). The control AgNO₃ solution color
remained unchanged and could not form the characteristic peak indicating that the reaction conditions were optimum with no abiotic reduction of AgNO$_3$. UV-Visible spectroscopy was used as a simple and reliable method for monitoring the stability of nanoparticle solutions. As the particles destabilize, the original extinction peak will decrease in intensity (due to the depletion of stable nanoparticles), and often the peak will broaden or a secondary peak will form at longer wavelengths (due to the formation of aggregates) (24). The UV-visible absorption spectrum of $H.\text{col.}$ AgNp is displayed in Figure 2. A strong absorption peak at approximately 450 nm originated from the surface plasmon resonance absorption of AgNp particles. The good symmetric absorption peak implies that the size distribution of the nanoparticles is narrow.

![Figure 1](image1.png)

**Figure 1.** (A) 1 mM silver nitrate solution with plant extract (*Hemigraphis colorata*)

(B) After 5 hours of incubation under dark conditions silver nanoparticles synthesized exhibit yellowish brown color.

**UV-Vis Spectral Analysis of Silver Nanoparticles**

![Figure 2](image2.png)

**Figure 2.** UV Visible spectrum of *H.col* AgNp.

TEM analysis micrograph (Fig.3a, b, c.) clearly show individual nanoparticles with sizes ranging from 20 to 44 nm with an average size of 23.0nm. The majority of the AgNPs were roughly circular in shape with smooth edges. The measurement of size was achieved along the largest diameter of the particles. Figure 3c shows the selected area electron diffraction (SAED) pattern.
obtained from AgNPs. The Scherer rings showed that the Ag NPs are scattered over the surface and no aggregates are noticed under the TEM. The difference in size is possibly due to the fact that the nanoparticles are being formed at different times.

3.2. Physical Characterization of GCG Scaffold incorporated with H.col. AgNp:

3.2.1. SEM Analysis

GCG Scaffold incorporated with H.col. AgNp were formed successfully using equal volume of chitosan and gelatin (w/v) and 0.5 % GAG with an addition of 1 % drug-loaded AgNp. The microstructure morphology of GCG AgNp scaffolds by cross-sectional SEM images was obtained (Fig.4). GCG Scaffolds confirmed a closed uniform network and pore configuration. SEM analysis showed a little increase in pore size in GCG H.col AgNp incorporated scaffold than GCG scaffold alone. Both scaffolds are formed without addition of any chemical cross linking agent. Freezing solutions at -20°C and lyophilization itself resulted in a uniform scaffold with an average pore size of 100 μm.

3.2.2. Swelling Behavior

The swelling studies of the GCG scaffold and GCG Scaffold with H.col. AgNp in PBS are shown in Fig. 5. Both scaffolds showed nearly constant degree of swelling ratio.
Fig. 5. The swelling ratio of GCG scaffold

The swelling ratio largely depends on hydrophilicity of used materials and their property to retain three-dimensional structure.

3.2.3. Radical-scavenging activity of scaffolds.
DPPH radical scavenging activity is a significant method to evaluate the antioxidant activity. GCG scaffold with incorporated herbal AgNp showed high-scavenging activity than GCG alone. This increased radical-scavenging activity is due to the effective antioxidant activity of incorporated herbal AgNp in the films. Radical-scavenging activity of films was assessed by comparison with ascorbic acid and expressed as the IC50 value (μg/ml), i.e., the concentration necessary to decrease the DPPH concentration by 50% where GCG Scaffold with H.col.AgNp had the highest free radical scavenging capability with an IC50 of 52.84 µg/ml.

3.2.4. Antimicrobial activity
GCG scaffold incorporated with H.col nanoparticles showed potent activity against Staphylococcus sp. and Pseudomonas sp. which may commonly worsen the burn healing process.

Figure 6. Antimicrobial activity of GCG scaffold incorporated with H.col.AgNp
3.2.5. MTT Assay

In order to evaluate the cytocompatibility of the GCG Scaffold and GCG Scaffold with H. col AgNP, the percentage of cell viability were quantified by the MTT assay. The MTT assay illustrated that the both scaffolds (4 x 10^4 cell/cm²) doesn’t have cytotoxic properties and have approving compatibility confirming this scaffold is excellent for dermal application. Biocompatibility of the scaffolds were tested by MTT assay with L929 cells, which revealed that there was no change in cell morphology and viability of L929 cells grown on contact showed higher proliferation (> 90 %) of the cells on the scaffolds with enhanced percentage viability in GCG Scaffold with H.col.AgNP (Fig.7a,b.).

![Graph showing cell viability in MTT Assay. GCG Scaffold showed 92% viability and GCG Scaffold with H.col AgNP showed 97% viability when compared with control.](image)

3.2.6. Evaluation of Wound healing potency

*In vitro* scratch assay was carried out to trail movement of individual cells in the leading periphery of the scratch. Analysis of the images showed that the cells in both migrated at fast rate compared with the control cells in the leading edge of the scratch.

The in vitro scratch assay is predominantly apt for studies on the effects of cell migration during wound healing in vivo. In addition to examine migration of homogenous cell populations, this technique has also been approved to quantify migration of individual cells in the chief frame of the scratch.

After creating the scratch (time point zero), a very short lag phase may take place until the cells begin drifting towards the scratch edge. The cells may directly enter into the linear growth phase, where the wound closure rate is invariable. When reaching confluency, the swiftness initially slows down and then goes to zero when the coverage attains 100%. Scratch assay confirmed that H.col AgNP incorporated in GCG scaffold accelerated closure of the gap in a time-dependent manner as the release of H.col AgNP, slightly increasing from 12^{th}, 24^{th} and 48^{th} hours after the scratch(Fig.8).
IV. Discussions

The focus of this study is to create and evaluate a scaffold dressing from fish discards for burns and wounds incorporated with nanoparticles of *H. col.* The characterization of *H. col.* AgNp, GCG scaffold and GCG scaffold incorporated with *H. col.* AgNp were carried out and the overall evaluation results correlated more or less with previously published reports (26, 27, 28). In addition, assessment of outcomes with published results (29, 30) pointed out superior cross linking even without addition of chemical cross linking agents.

The transfer of colorless AgNO3 solution turned yellow to brown or reddish yellow to deep red, indicated the formation of AgNPs having l max values which was reported earlier in the visible range of 400-500nm (31). The inhibitory action of silver on bacterial cells is related to the strong interaction of silver with thiol groups present in key respiratory enzymes in bacteria (32). Nano crystalline silver shows the most effective inhibitory action with a rapid inhibition rate (33). In our experiment, when we compared the antibacterial activity of GCG scaffold with and without *H. col.* AgNPs, it was found that GCG scaffold with *H. col.* AgNPs have shown more antibacterial activity.

In this study, a novel ‘GCG scaffold incorporated with *H. col.* AgNp’ for burn wound healing was evaluated. Biocompatible and biodegradable biopolymers like glycosaminoglycan, chitosan and gelatin were used to fabricate scaffolds by freezing and lyophilizing methods. The enrichment of chitosan with thiol-modified gelatin enhances its biological properties (34). The scaffolds made in this study had an almost symmetric structure as SEM analysis reveals. There are further connective and larger pores available in this scaffold. Swelling ability tests exhibited that the scaffold is revealing elevated hydrophilicity. Thickness, pore size, and pore distribution of the scaffold are the significant factors for cell growth and drug transit in the scaffold. Both chitosan and glycosaminoglycans have an abundant number of hydrophilic groups, such as hydroxyl, amino, and carboxyl groups, which can promote water uptake in the structure (35). The water absorption property of the raw materials influences not only the maintenance of scaffold’s shape but also affects the cells growth. Swelling behavior and structural stability of scaffolds enhances its use in tissue engineering. Most natural polymers, containing gelatin, chitosan, swell readily in biological fluids. In this study, the shape of the membrane was flexible rather than fragile in nature. This phenomenon points out that GCG scaffold could maintain stable swelling ratio.
The presence of amine and hydroxyl groups in chitosan molecules facilitate the formation of hydrogen bonds, leading to the crystalline structure of the polymer (36). As chitosan and glycosaminoglycans have an opposite charge, an ionic bond between the carboxyl groups of glycosaminoglycans and the amino groups of chitosan is expected (37).

The morphological appearance of the GCG scaffold is standard with an average porous diameter of 100 μm (Figure 3a) and high porosity (void-volume fraction) 99.0% (± 0.03%). Compared with porosity of GCG control matrix, H.col.AgNp incorporated GCG scaffold matrix porous diameter have slightly increased. These values come under the permitted range used for scaffolds in tissue engineering. Porous sizes below 20 μm would not allow free access to the skin cells, and pores with mean sizes greater than 125 μm would not allow proper cell attachment (38). Literature indicated that porosities greater than 95% are appropriate for tissue engineering (39).

Antioxidant effectiveness is related to activation energy, rate constants, oxidation–reduction potential, ease with which the antioxidant is lost or destroyed (volatility and heat susceptibility), and antioxidant solubility (40). Studies have shown that the Hemigraphis species possess very good antioxidant activities and contain phenolic compounds which are effective hydrogen donors that make them good antioxidants (41).

The results of in vitro cytotolerance by MTT assay authenticate that this GCG scaffold is an excellent matrix for dermal regeneration which aids in burn wound healing. Evaluation of wound healing potency by scratch wound assay ensured the wound healing activity of the scaffold under study. The in vitro scratch assay images captured at the beginning and at regular intervals during cell migration exposed the activity of GCG scaffold and GCG scaffold incorporated with H.col.AgNp to close the scratch and comparing the images to quantify the migration rate of the cells. Compared to other methods (42, 43) the in vitro scratch assay is mostly appropriate for learning the effects of cell-matrix and cell migration during wound healing in vivo. Altogether, we suggest that this GCG scaffold with H.col.AgNp incorporated have distinctive structure, which promotes the wound healing process by accelerating disintegration of the scaffold and H.col. as a drug enhancing wound healing from H.col.AgNp during the repair phase.

V. Conclusion

The incorporation of H.col AgNp (5%) in GCG scaffolds improved the biological properties of GCG scaffolds and provides a highly hydrated, pericellular environment that simulates the ECM. The combination of glycosaminoglycan with gelatin and chitosan has improved the pore network configuration, pore size, mechanical properties and swelling ratio. The biological functions of GCG scaffolds seem to be influenced by physicochemical factors, such as pore network configuration, pore size, swelling ability, and mechanical strength to reconstruct the dermis. The analysis of GCG scaffold suggests that the freeze-dried scaffolds can constitute a suitable bed for further regeneration of dermal and epidermal tissues and play a pivotal role in burns and wound healing. In this growth-favorable background, gathering of other matrix machinery and emergence of growth and differentiation factors and cell migration contribute to its potential use in the accelerated burns and wound repair.

Bibliography


