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RESEARCH ARTICLE

Preparation and Characterization of Gellan Gum Hydrogel as Therapeutic Protein Delivery for Wound Healing

Ahmad Rasul Radzali[a], Chen Hui Cheng[b], Rozanaliza Radzi[a], Michelle Fong Wai Cheng[a], Adila Mohamed Jaafar[c], Pei-Shue Jason Tsai[d], Mokrish Ajat[e,f*]

[a]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; [b]Department of Companion Animal and Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; [c]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; [d]School of Veterinary Medicine, National Taiwan University (NTU), Taipei, 10617, Taiwan; [e]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; [f]Natural Medicines and Natural Product Research Laboratory (NaturMeds), Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract Therapeutic proteins like platelet-rich plasma have been used as adjunct therapies for wound healing. The delivery of these proteins may require a special carrier as a controlled release to prolong and optimize the healing effects on the affected tissues. The present study focuses on preparing and characterizing a hydrogel made from gellan gum to act as a scaffold to carry therapeutic proteins intended for wound healing. Fetal bovine serum (FBS) was used as a representative for therapeutic proteins due to its ability to stimulate cell proliferation in vitro. FBS, gellan gum (GG) hydrogel, and FBS-loaded gellan gum hydrogel (GF) were prepared and characterized by the detection of its functional groups through FTIR and elemental analysis through CHNS analyzer, confirming the entrapment of biomolecules of FBS into GG. The protein release study showed a burst release of protein from all GF variants with subsequent gradual slow release over 72 hours period. Cell viability (MTT) assay showed an increasing trend of cell viability percentage with the increasing concentration of FBS loaded into GG hydrogel. The results of this study support the potential use of GG hydrogel as a carrier of therapeutic proteins for wound regeneration.

Keywords: Gellan gum; hydrogel; therapeutic protein; scaffold; wound healing.

***For correspondence:**
mokrish@upm.edu.my

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Introduction

Wound healing has been a frequent focus of studies in medical and veterinary fields due to its clinical, scientific, and economic concerns [1,2]. The process of repairing a wound can be quite complex, as it involves the movement of inflammatory cells and collagen, the effects of cytokines, the formation of extracellular matrix, and scar remodelling. Nevertheless, if the skin's ability to regenerate is impaired, the wound may not heal for an extended period and become chronic [3]. Chronic wounds are harder to manage than acute wounds as they heal more slowly, persistently, and abnormally, thus, could result in serious consequences that necessitate tissue excision, and increased costs of treatment [4]. Hence, adjunct therapies like platelet-rich plasma, which supplies therapeutic proteins such as growth factors, may be considered after all efforts have been taken to treat the wound, yet there is still inappropriate wound healing progression [5].

In delivering these protein-based therapeutics onto the wounds, a special delivery system may be required to be incorporated as it does not only prevent these proteins from early degradation, it also may act as a controlled release to prolong and optimize the healing effects on the affected tissues [6]. A well-known type of scaffold for protein delivery is hydrogel [7]. Hydrogel is a three-dimensional network of hydrophilic polymers that can swell to an equilibrium state in the presence of water or biological fluids [8]. It does not only provide a moist environment that gives soothes wounds and resembles the one in which cells grow [9] but it can also be tuned to obtain different shapes, porosity, swelling capability, and cross-link density that can aid in its property to control release certain therapeutic agents [7,10]. Depending on the composition of polymers used to create them, hydrogels can be categorized as natural, synthetic, or hybrid [11].

Gellan gum (GG), a polysaccharide synthesized by a non-pathogenic gram-negative bacterium called Pseudomonas elodea, is one of the naturally occurring polymers that can be made into a hydrogel [12]. GG hydrogel is known to be biocompatible and able to change its mechanical properties and degradation profile depending on the degree and types of crosslinking it contains [13]. The use of GG hydrogel has attracted a lot of interest to be a potential carrier in controlled release studies [14]. It has been used as a drug release system and combined with materials such as collagen, stem cells, antimicrobials (eg. ofloxacin), lavender oil, and others, for wound healing purposes [15]. The combination of GG with therapeutic proteins, like platelet derivatives, has been found to enhance the micro-environment for chondrogenesis of adipose-derived stem cells in vitro [13]. Hence, the use of GG hydrogel may be worth to be investigated as a potential scaffold for therapeutic proteins to provide an alternative for wound regeneration.

This study aims to prepare and characterize GG hydrogel as a potential scaffold for therapeutic proteins for skin wound regeneration. Fetal bovine serum will be used in the study preliminarily as a source of therapeutic proteins, prior to the use of other therapeutics like platelet-rich plasma, as it is known to have biomolecules and protein components that can promote cell growth [16] (Kwon 2016).

Materials and Methods

Materials

Gellan gum powder was purchased from Alfa Aesar (Ward Hill, MA, US). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Antibiotic-Antimycotic (100X), Trypsin -EDTA (1X) 0.25%, Fetal Bovine Serum, Bicinchoninic (BCA) Protein Assay Kit, and Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12(1:1), L-glutamine, 15mM HEPES) were purchased from Thermo Fischer. Phosphate buffered saline was made by dissolving associated salts purchased from Chemiz (Malaysia) in distilled water, with adjusted pH of 7.4 before autoclaving. Normal human dermal fibroblast (NHDF) cells were obtained from Tissue Engineering Centre, UKM Medical Centre, Malaysia.

Preparation of GG Hydrogel Loaded with FBS

GG hydrogel was prepared by dissolving 0.2g GG powder in 10ml distilled at 90ºC using a magnetic stirrer with a heater for 30 minutes, then the heater temperature was reduced to 65ºC for the next 30 minutes. The dissolved solution was then poured into 90mm Petri dishes for it to solidify for 10 minutes at room temperature. Then, the GG hydrogel was kept at 4ºC chiller for 1-2 days prior to use. The inclusion of FBS into the GG hydrogels was done by submerging cut GG hydrogel with various concentrations of FBS solution overnight inside a 37ºC incubator prior to the protein release study or MTT assay.

Fourier-Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to detect the presence of the functional groups in FBS as well as GG hydrogel with and without the FBS, using a Bruker INVENIO® R FTIR Spectrometer. All samples were prepared using the UATR technique and analyzed in the region of 4000 to 400 cm^{-1}.

Elemental Analysis

Elemental analysis was performed to determine mass fractions or the amount of carbon (C), hydrogen (H), and nitrogen (N) in the samples using a LECO TruSpec® CHNS Analyzer instrument. Each finely freeze-dried sample was weighed (1.9-2.1 mg) in a tin container. This container was then combusted in a reactor at a temperature of 1000°C. The products produced from the combustion were reported in weight percentages. The data from the combustion analysis of GG, FBS, and GF (FBS-loaded gellan gum hydrogel) used were recorded.

Protein Release Study

A protein release study was done on GG hydrogels incorporated with fetal bovine serum (FBS) by submerging 8mm diameter GG hydrogel discs (cut by using punch biopsy) with various concentrations of FBS diluted in phosphate-buffered saline (PBS) at 100% (GF100), 75% (GF75), 50% (GF50), 25% (GF25) and 0% (GF0) in the wells of 24 well plate overnight. The next day, the solution from each well was removed and replaced with 2ml of PBS. The plates were shaken at 100rpm on an orbital shaker at room temperature. At time points of 1h, 6h, 24h, 48h, and 72h, 0.5 ml of the total solution from each well was withdrawn at kept at -20ºC in 1.5ml microcentrifuge tubes. 0.5 ml of fresh PBS was refilled after each withdrawal. At the end of the study, the withdrawn solutions were subjected to Bicinchoninic acid (BCA) assay to analyze the protein concentration release.

Cell Culture

The cells were cultured in cell culture flasks in DMEM/F12 media supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Media was changed every 2-3 days cells reaching confluency before use. Cell passages 4 to 6 were used in this study.

Cell viability Assay

MTT assay on dermal fibroblasts treated with GG hydrogel was done by first placing 4mm hydrogel discs that were cut by using a punch biopsy into the wells of 96 well plates. Then, 100µl of the solution containing 100%, 75%, 50%, 25%, and 0% FBS diluted with DMEM/F12 media was added into the wells containing the GG hydrogel disc to prepare the hydrogels labelled GF100, GF75, GF50, GF25, and GF0 respectively. The plates were incubated in a 37ºC incubator overnight to allow the incorporation of FBS into the GG hydrogel. The next day, after removing the previous solution, 10,000 cells were seeded into each well. The plates were incubated for 24h, 48h, and 72h in 37ºC and 5% CO2 incubator. At selected time points, the old media was replaced with MTT-containing media before being further incubated for 4h which was then replaced by adding a DMSO solution. The plates were shaken on an orbital shaker at 100rpm for 20 mins. Then, the dissolved solution from each well was transferred into a new empty well for absorbance reading at 570nm.

Statistical Analysis

All tests were done in 3 replicates. The data were analyzed using the GraphPad Prism 7.0 software (GraphPad Software) with 2-way ANOVA for protein release study and Kruskal-Wallis test for MTT assay. P values of less than 0.05 were considered statistically significant.

Results and Discussion

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Figure 1 shows the spectra of FTIR for GG, FBS, and GF. In general, GG showed absorptions bands at 3318 cm^{-1} (hydrogen-bonded OH, hydroxyl stretching), 2963 cm^{-1} (C-H, alkane stretching), 1603 cm^{-1} (C=O, carbonyl stretching), 1410 cm^{-1} (CH3, methyl bending), 1260 cm^{-1} / 1013 cm^{-1} (C-O stretching) and 795 cm^{-1} (=C-H, alkene out-of-plane bending). The FTIR finding of the GG was found to be quite similar when compared to a previous study that analyze GG [17]. On the other hand, FBS displayed peaks at 3273 cm^{-1} (OH stretching), 2930 cm^{-1} (C-H stretching), 1636 cm^{-1} (C=O stretching), 1537 cm^{-1} (N-H bending), 1397 cm^{-1} (CH3 bending), 1032 cm^{-1} (C-O stretching). The absorption peaks in FBS especially at 1636 cm^{-1} (C=O stretching) and 1537 cm^{-1} (N-H bending), may indicate the presence of an amide group that might come from the protein components contained in FBS. This finding was also similar to an FTIR of a plasma sample that showed two peaks around 1650cm^{-1} (primary amide, C=O stretching vibration), and 1547cm^{-1} (secondary amide, N-H bending and C-N stretching) [18].

Based on the absorption spectra result, it was found that all the peaks belonging to FBS were present in GF with slight shifting, indicating the successful incorporation of FBS into GG. The peaks in GF were present at 3275 cm^{-1} (OH stretching), 2930 cm^{-1} (C-H stretching), 1636 cm^{-1} (C=O stretching), 1541 cm^{-1} (N-H bending), 1395 cm^{-1} (CH3 bending), 1240 cm^{-1} (C-O stretching) and 1059 cm^{-1} (C-O stretching). Due to perceived interactions between GG and FBS, some of the bands present in GG may have appeared less intense, shifted, or disappeared in GF.

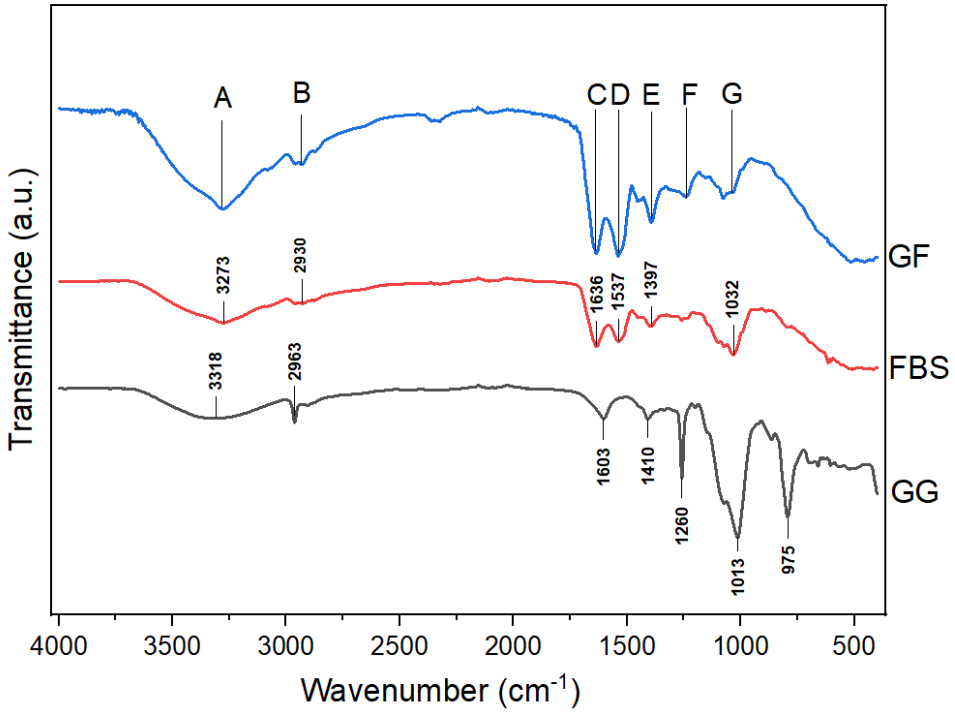

Figure 1. Fourier Transform Infrared Spectroscopy (FTIR) analysis of gellan gum hydrogel (GG), fetal bovine serum (FBS), and fetal bovine serum-loaded gellan gum hydrogel (GF) in the wavelength range of 400-4000nm^{-1}.

Table 1. List of functional groups and absorption peaks for fetal bovine serum-loaded gellan gum hydrogel (GF)

Symbol	Functional Group	Absorption peak (cm^{-1})
A	O-H broad	3275
B	C-H stretch	2930
C	C=O stretch	1636
D	N-H bend	1541
E	CH3 bend	1395
F	C-O stretch	1240
G	C-O stretch	1059

Elemental Analysis

Elemental analysis was done to analyze the carbon (C), hydrogen (H), and nitrogen (N) content in GG hydrogel, fetal bovine serum (FBS), and fetal bovine serum-loaded gellan gum hydrogel (GF). Based on Table 2, the C, H, and N contents of GG were 32.25%, 5.46%, and 0% respectively. This is expected as GG it is a type of polysaccharide [13], which generally consists of elements C, H, and O [19,20]. Meanwhile, FBS showed 40.75% C, 5.460% H, and 5.321% N. The higher nitrogen content in FBS is most probably due to the presence of various protein components that it contains [21].

Although FBS showed a measurable nitrogen percentage from the elemental analysis when incorporated into GG hydrogel, the nitrogen percentage was found to be below the detection limit as was seen in GF. As CHNS analysis corresponds to the mass fraction of the elements [22], it can be said that the nitrogen content contributed by the FBS has formed a very low mass faction below the detection limit, after being incorporated into GG hydrogel. With that being said, the C and H weight percentages of GF that were

found to be higher than GG have become the evidence that supports the successful incorporation of FBS into the GG hydrogel.

Table 2. Elemental analysis of weight percentage of carbon (C), hydrogen (H), and nitrogen (N) in gellan gum hydrogel (GG), fetal bovine serum (FBS), and fetal bovine serum-loaded gellan gum hydrogel (GF).

Material	Weight Percentage (%)		
	C	H	N
GG	35.25	5.233	0
FBS	40.75	5.460	5.231
GF	36.48	5.487	0

Protein Release Study

The protein release study is important to show that proteins incorporated into the GG hydrogels are being released in a sustained manner so that they can exert their therapeutic effects on the target of interest. The release of protein from the GG hydrogel is hypothesized to be either from dissociation of the molecules inside the hydrogel followed by diffusion through it, or by release of the molecules from the hydrogel [23].

Figure 2 displayed the protein concentration released from different GG hydrogel variants over 72 hours period. From the graph, it is shown that there was a burst release of protein initially at 1h for all GG hydrogel variants, and then the protein release gradually reduced over the 72h period. The initial burst release pattern was also found to be similar to previous research that used GG-based hydrogels that were incorporated by certain drugs [23,24] and can be explained by the faster release of the molecules adsorbed at the hydro-gel surface than the ones incorporated inside the hydrogel [25].

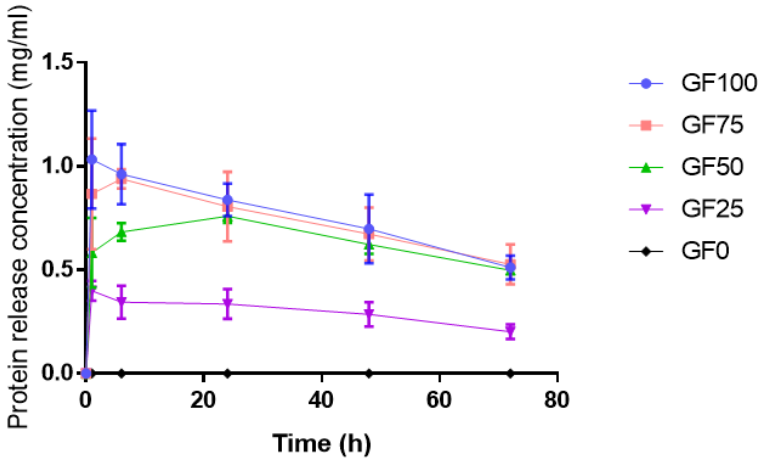

Figure 2. Protein release kinetics study of GG hydrogel incorporated with different concentrations of fetal bovine serum (GF0 – GF100), respectively. Experiments were completed in at least triplicates and the values were expressed as protein concentration ± SD.

GF100 showed the highest protein concentration release at almost all time points suggesting higher protein molecules incorporated into the GG hydrogel prior to being released. On the other hand, GF25 showed significantly lower protein release concentration when compared with that of GF100. From this finding, it can be said that, when a higher protein concentration is loaded into the GG hydrogel, a higher protein concentration will be released, followed by a gradual release of protein over 72 hours period. This protein release study supports the potential of GG hydrogel to carry and sustain the release of therapeutic proteins.

Effect of FBS-Loaded GG Hydrogel on dermal fibroblasts

The effects of different GG hydrogel variants on the cell viability of dermal fibroblasts were analyzed by

using an MTT assay (Figure 3). Based on the graph, there is an increasing trend of cell viability % with the increasing concentration of FBS used in the GG hydrogel, particularly on day 3. The increased cell viability was found to be statistically significant particularly in those treated with GF100 and GF75, when compared with GF0, after 72 hours. This could indicate a higher release of bioactive components of FBS from the GF100 that could positively affect cell proliferation and thus, cause an increase in cell viability.

Although the negative control showed the highest cell viability % on days 1 and 2 post-seeding, this finding can be explained by the limited space available that the cells can attach to during seeding caused by the initial hydrogel placement. However, over time, cells were seen to be growing underneath the hydrogels leading to increased cell viability, especially in those wells incubated with GG hydrogel incorporated with a higher concentration of FBS. The increased cell viability effect on cells over time was also observed in previous research that used the combination of gellan gum and other therapeutic protein source like platelet-rich plasma [13].

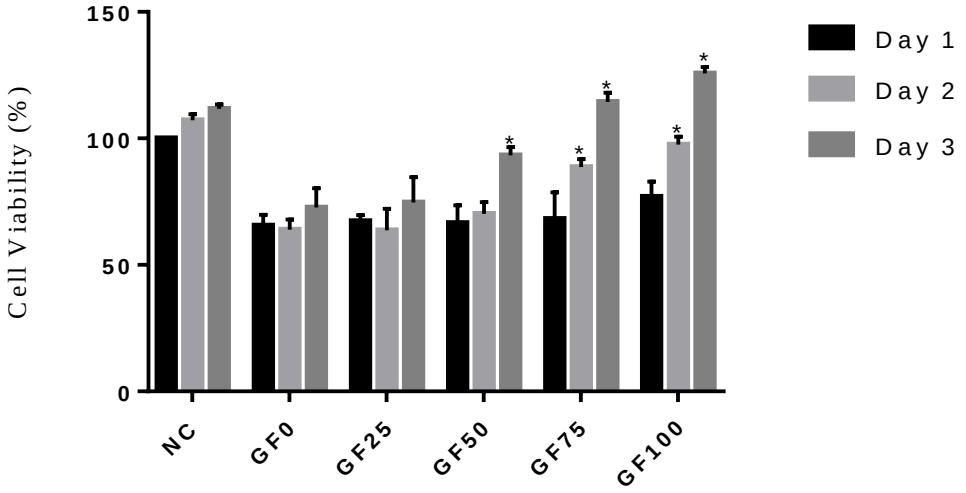

Figure 3. Cell viability percentages by MTT assay of dermal fibroblasts cultured with GG hydrogels incorporated with several concentrations of fetal bovine serum (GF0 – GF100), respectively. NC refers to negative control which used serum-free media. Experiments were completed in at least triplicates and the values were expressed as mean percentage cell viability ± SD. * $p < 0.05$ when compared with GF0 at the same time point.

Conclusions

From these in vitro studies, it was found that GG hydrogel has the potential to become a suitable delivery system that can carry therapeutic proteins and release them in a sustained manner to increase the cell viability of dermal fibroblasts. The use of other therapeutic proteins like growth factors or platelet-rich plasma that are known to have bioactive healing components is suggested to be incorporated into the gellan gum hydrogel so that it may be used in future studies that aim for wound regeneration. From these in vitro studies, it was found that GG hydrogel has the potential to become a suitable delivery system that can carry therapeutic proteins and release them in a sustained manner to increase the cell viability of dermal fibroblasts. The use of other therapeutic proteins like growth factors or platelet-rich plasma that are known to have bioactive healing components is suggested to be incorporated into the gellan gum hydrogel so that it may be used in future studies that aim for wound regeneration.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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