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Synthesis and characterisation of glutaraldehyde cross-linked κ-carrageenan-gelatin hydrogel

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Abstract
Hydrogels are three dimensional hydrophilic networks composed of either homopolymeric or heteropolymeric chains, which have the ability to absorb high amount of water and drugs within its polymeric structure without itself getting dissolved in water. A Carrageenan-Gelatin hydrogel was prepared by dissolution of the dry carrageenan and gelatin powder in distilled water followed by heating and stirring until a homogenous solution was obtained. The solution was solidified and dried at room temperature to a constant weight. The film was cross-linked by immersing in 5% glutaraldehyde solution in presence of 0.1N HCl and was cured at 110^0C for 25 minutes and air dried at room temperature to a constant weight. The swelling index (1801.385 ± 94.31%) of the obtained hydrogel indicated that carrageenan was polymerized. The physical characterization of hyudrogel revealed that gel fraction was 53.99 ± 0.14%, water absorption percent was 1701.385 ± 94.31% and equilibrium water content was 94.38 ± 0.27%. It was also found that the gel do not permit bacteria to penetrate though it. The obtained hydrogels have the potential for biomedical application as vehicle.

Keywords: Hydrogel, carrageenan, gelatin, glutaraldehyde cross-link, gel characteristics

1. Introduction
In recent centuries, much consideration has been concentrated on the research and development of polymer hydrogels for wound dressing and drug delivery systems. Polysaccharides, as natural biomolecules, are obvious choice as a potential wound dressing substitutes [1]. The terms gels and hydrogels are used interchangeably by food and biomaterials scientists to designate polymeric cross-linked network configurations. Gels are extensively dilute cross-linked systems that are characterized as weak or strong depending on their rheological properties in steady-state [2]. Hydrogels are synthesized by physical methods such as heating and cooling, repeated freezing and thawing, irradiation with electron beam or γ-rays or chemical methods using a covalent cross-linking agent including boric acid, formaldehyde and glutaraldehyde[3]. The hydrophilic functional groups stick to the backbone of the polymer facilitate the hydrogels to absorb water. The cross-links between the network chains delivers them with the property of resistance to disintegration [4].
Many biodegradable polymers like alginate [5] chitosan [6], carrageenan [7], gelatin [8], polyvinyl alcohol [9] etc. have been used for making hydrogels. In current work, we have chosen two natural polymers, gelatin and κ-carrageenan for the preparation of hydrogel. Choosing gelatin as an alternative to other polymers was due to its properties of being non-toxic, non-immunogenic, biodegradable and reasonable price. The benefit of using gelatin over collagen involves its inexpensiveness and high dissolving capability in water. The gels formed from gelatin are naturally biodegradable and show no cytotoxicity toward human cells [10]. κ-carrageenan is a natural polysaccharide that is obtained from edible red seaweeds of the class Rhodophyceae. It has been reported that the carrageenan have high capability to form thermo-reversible gel due to which they can be used for the controlled release of drug [11].
Chemically cross-linked gels are true or permanent hydrogel with covalent bonds. The ability to swell and degree of swelling of hydrogels are mainly governed by two features, namely the hydrophilicity of polymer chains and the crosslink density [12]. Current study deals with the formation of permanent hydrogel network by glutaraldehyde cross-linking. Aldehydes are very reactive and have been used for crosslinking polymers among which glutaraldehyde is efficient, easily available and inexpensive.
Hydrogel of many synthetic and natural polymers have been produced with their end use mainly in tissue engineering, pharmaceutical, and biomedical fields [13]. Hydrogels have many other benefits, such as non-toxicity, non-adherence and smoothness and hence are very suitable to be used as wound dressings material [14].

Wounds covered with hydrogel dressings showed faster wound healing and formation of well-developed fibroblast and blood capillaries compared to open wound [15].
Current study investigates the characteristics such as gel fraction percentage, swelling index, water absorption percentage and equilibrium water content of carrageenan-gelatin hydrogel. *In vitro* microbial penetration was performed to confirm the protection afforded by hydrogel on the wound from invading microorganisms from the external environment.

2. Materials and Methods

2.1 Materials used

κ- carrageenan and gelatin powder for hydrogel synthesis was procured from M/s Himedia Laboratories Pvt. Ltd. India and Nice Chemicals (P) Ltd. India, respectively. Cross-linking agent g02lutaraldehyde (25%) and hydrochloric acid were procured from Merck Life Science Pvt. Ltd. Mumbai.

2.2 Synthesis of hydrogel film

Carrageenan-Gelatin hydrogel was prepared by dissolution of the dry carrageenan and gelatin powder in distilled water. The mixture was heated and stirred until a homogeneous solution was obtained. The solution was poured into glass petri plate and allowed to solidify and dried at room temperature to constant weight.

For standardization of hydrogel film different concentrations of carrageenan and gelatin powder were mixed in multiple proportions (1:1, 1:2, and 1:3) as given in the table 1.

Table 1: Proportions of gelatin and carrageenan used for preparation of hydrogels

Formulations	Proportions
F1	1% Gelatin + 1% κ-carrageenan
F2	1% Gelatin + 2% κ-carrageenan
F3	1% Gelatin + 3% κ-carrageenan

1% (w/w) of gelatin stock solution was prepared by dissolving the gelatin granules in the distilled water at 60 ºC with constant stirring in water bath for 10 minutes. 1%, 2% and 3% (w/w) of κ-carrageenan solution was prepared by dissolving the whitish powder of carrageenan in the distilled water at 90 ºC with constant stirring in water bath for 20 minutes. The optimization of the formation of gels was carried out by trial and error method. The two solutions were taken in different ratio with increasing concentration κ-carrageenan and gelatin concentration as constant.

2.3 Hydrogel film cross-linking

2.3.1 Film immersion and curing

5% Glutaraldehyde was prepared by diluting 25% glutaraldehyde with distilled water. The carrageenan-gelatin film was immersed in 5% glutaraldehyde and 0.1N HCl mixture in the ratio of 3:1 (cross-linker) for 4 min. The surface of film was wiped with cloth and then cured at 110^0C in hot air oven for 25 min. The cross-linked film was soaked in water with stirring in rocker shaker for 1 min and then in propanol for overnight to remove unreacted glutaraldehyde. The wet hydrogels were dried at room temperature to a constant weight [11]

2.4 Film characterization

2.4.1 Physical charecterisation

A. Gel Fraction Percentage

The pieces of hydrogel samples (2×2 cm) were dried for 6 h at 50 °C (W_0). They were soaked in 10 mL distilled water into petri dishes for 24 h up to a constant weight and taken out from petri dishes in order to remove the soluble parts [3]**.** The gels were dried again at 50 °C (We). The gel fraction percentage was calculated by the following equation:

$$\text{Gel fraction\%} = (We / W_0) \times 100$$

Where W_0 and We are the weights of hydrogel samples dried for 6 h at 50 °C before and after soaking, respectively.

B. Swelling Measurement

The pieces of hydrogel samples (2 cm × 2 cm) were dried at 60 ºC for 12 h (W_d), then soaked in pH 7.4 phosphate buffer solution (PBS) at 37 ºC (Ws). The swelling ratio (SR) was calculated using the following equation [16].

$$SR\% = (Ws / Wa) \times 100$$

Where W_d and Ws are the weights of hydrogel samples dried for 12 h at 60 ºC and soaked in PBS at 37 ºC respectively.

C. Water Absorption Percentage

The hydrogel samples were immersed in distilled water for regular intervals of time at 37^OC. After the excessive surface water was removed with filter paper, the weight of swollen gel was measured until there was no further weight increase. The water absorption percentage was determined according to the following equation: [17].

$$\text{Water absorption\%} = (Ws\text{-}W_d)/ W_d \times 100$$

Where Ws is the weight of swollen sample and W_d is the weight of dried sample

D. Equilibrium Water Content

The water absorbed by hydrogels is quantitatively represented by the equilibrium water content (EWC) as mentioned above for water absorption percentage. Equilibrium water content was measured by the equation: [18].

$$\text{Equilibrium water content (\%)} = (W_S - W_d) / W_S \times 100$$

Here, W_S is the mass of the swollen gel at time t (equilibrium) and W_d is the mass of the dry gel at time 0.

2.4.2 Biomedical property- Microbial penetration assay

The ability of hydrogel film to prevent microbial penetration was tested by attaching polymeric film to the top of glass test tube containing 5 ml of sterile nutrient broth. Before test, polymeric films, nutrient broth and glass test tubes were sterilized. Polymeric films were sterilized by treating them with 100% ethanol for 10–15 minutes and exposed to UV light for 15 minutes. Sterile nutrient broth in glass test tubes closed with cotton ball was used as negative control and sterile nutrient broth in open air used as positive control. Wet the surface of the hydrogel film to create a similar condition in the wound environment using tap water. Microbial penetration was analyzed in terms of the development of cloudiness of nutrient broth after one month. All the studies are carried out in triplicate [15].

3. Results and Discussion

3.1 Synthesis of cross-linked hydrogel film

A series of glutaraldehyde cross-linked carrageenan-gelatin hydrogels having different proportions (Table-2) were

synthesized by film immersion and curing reaction. Physical appearance of synthesized carrageenan-gelatin hydrogels (glutaraldehyde as crosslinker) was observed as a light yellow in colour. For the fabrication of hydrogel film the concentrations of crosslinkers and polymers were optimized in order to obtain an ideal gel for wound dressing applications. Results of the carrageenan and gelatin at various proportions are given in table 2. Final optimized concentration for carrageenan 3% (W/V) and gelatin 1% (W/V) was established to three part and one part respectively; concentration of glutaraldehyde was optimized to 6% of total solution from glutaraldehyde stock solution (5%) and 0.1N HCl as catalyst (3:1). The appearance of this gel before cross linking and after cross linking and drying is given in Figure 1.

Table 2: Permutations of gelatin and carrageenan and quality of resultant hydrogel.

Formulations	Sample code	Gelatin	Carrageenan	Remarks
F1	F1a	1 mL(1%)	1 mL(1%)	No gel formation
	F1b	1mL(1%)	2 mL (1%)	No gel formation
	F1c	1 mL(1%)	3 mL(1%)	Gel formed but very thin
F2	F2a	1mL(1%)	1mL (2%)	Gel formed, Very thin
	F2b	1mL(1%)	2mL (2%)	Gel formed, Very thin
	F2c	1 mL (1%)	3 mL (2%)	Gel formed, Thin
F3	F3a	1 mL (1%)	1 mL (3%)	Gel formed, Thin
	F3b	1 mL (1%)	2 mL (3%)	Gel formed, Moderate thickness, less gel strength
	F3c	1 mL (1%)	3 mL (3%)	Gel formed, Moderate thickness, good gel strength

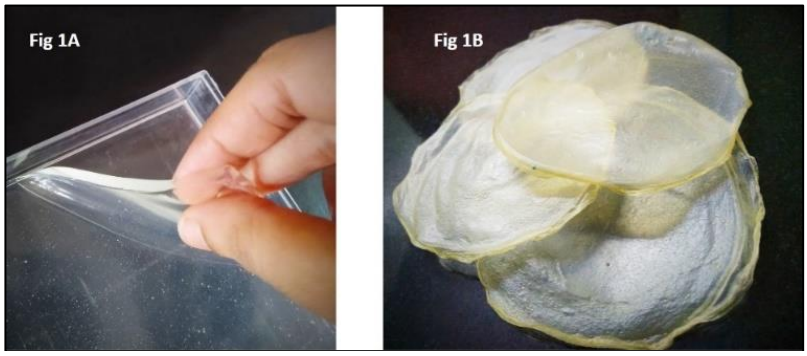

Fig 1: Consistency and appearance of hydrogel (F3c) formed by crosslinking 1% gelatin and 3% carrageenan (1:3) A. Gel before crosslinking B. After cross linking and drying.

3.2 Physical Characterization of hydrogel film

Physical charecterisation of prepared hydrogels was performed and the results are expressed in Table 3. The mean ± SEM of physical characteristics are given in Figure 2.

A. Gel Fraction Percentage

Gel fraction percent is the measure of dimension stability of the hydrogel film. The non-gel portion of hydrogel will be dissolved in water and the remaining residue was the actual gel portion. In this study the gel fraction percentage of cross-linked κ-carrageenan-gelatin hydrogel was 53.99 ± 0.14 per cent**.** This means that the hydrogel contain more than 50% of gel portion.

B. Swelling Ratio

The prepared hydrogel was characterized for its swelling property in aqueous medium. Ability of dry hydrogel to absorb water indicates that hydrogel based on carrageenan-gelatin was reversible. The maximum swelling index was in the range of 1600-2200 with a mean of 1801.385 ± 94.31 percent indicating that the hydrogel have a high potential to absorb water.

C. Water Absorption Percentage

The water absorption percentage of the hydrogel samples increased with increasing immersion time and then reached to almost equilibrium level.it ranges 1500-2100 percent with a mean of 1701.385 ± 94.31 per cent within one hour.

D. Equilibrium Water Content

Carrageenan-gelatin hydrogel have a maximum equilibrium water content of 94.38 ± 0.27% indicating that the hydrogel have a high potential to prevent wound bed from accumulation of exudate and also provide a moist environment.

Table 3: Physical charecterisation of Carrageenan-Gelatin hydrogel

Sample	Gel fraction (%)	Swelling Ratio (%)	Water absorption (%)	Equilibrium water content (%)
1	53.76	2193.73	2093.73	95.44
2	53.77	1754.58	1654.58	94.3
3	53.99	1959.86	1859.86	94.9
4	53.73	1612.71	1512.71	93.8
5	54.6	1632.23	1532.23	93.87
6	54.11	1655.2	1555.2	93.96
Mean ±SEM	53.99 ± 0.14	1801.385 ± 94.31	1701.385 ± 94.31	94.38 ± 0.27

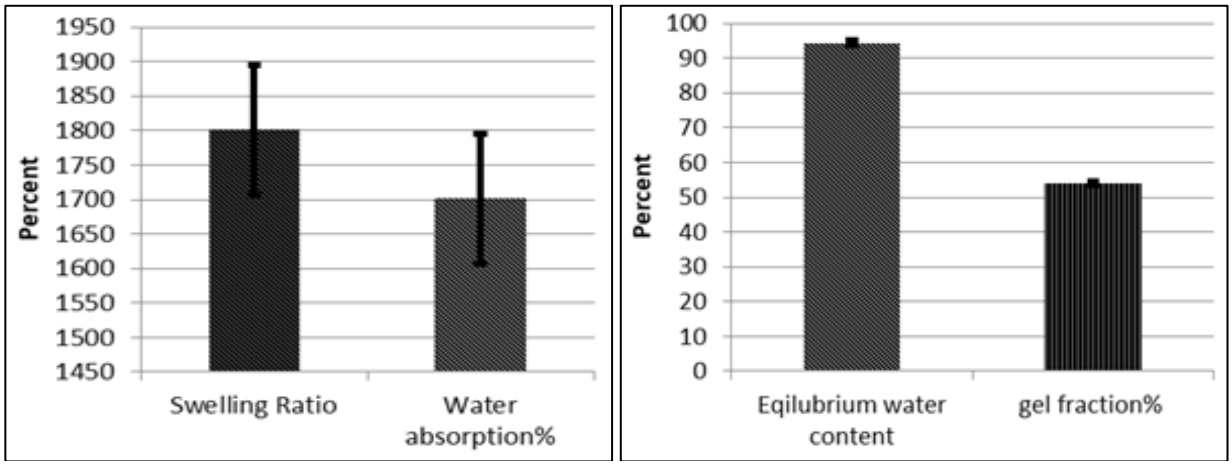

Fig 2: Mean (±SEM) physical characterization of Carrageenan-Gelatin hydrogel (n=6)

3.2.3 Biomedical properties- Microbial Penetration assay

The result of microbial penetration assay is depicted in Figure 2. The results showed that only the positive control test tube had bacterial contamination, whereas, neither negative control nor the carrageenan-gelatin hydrogel film have shown visible microbial penetration during one month period. Microbial impermeability of film will reduce the chance of secondary bacterial infection in the wound making more suitable for wound dressing.

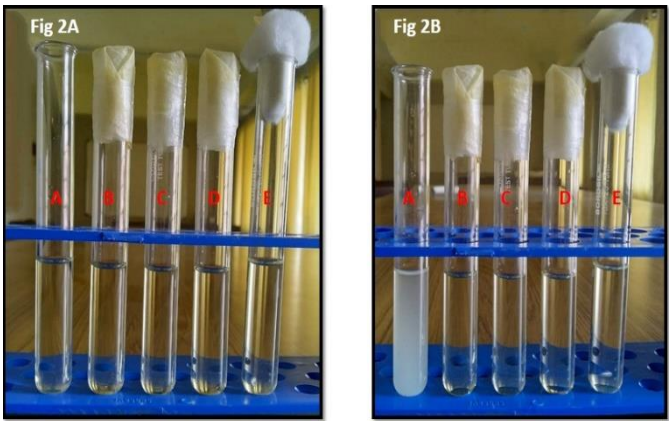

Fig 2: Microbial penetration assay for prepared hydrogel film. Fig 2A. first day of experiment Fig 2B. 30th day of experiment. (A-Positive control, B,C&D- Test and E- Negative control)

In this study glutaraldehyde crosslinking of κ-carrageenan-gelatin hydrogel improves the physical characteristics by crosslinking. Chemical crosslinking is a direct reaction between linear polymer or branches and at least a bifunctional component, small molecular weight, and called as crosslinker which links the polymer chains with its functional groups. Crosslinking is important because the unreacted carrageenan is still easily soluble in water. Thus crosslinking will improve the stability of hydrogel in aqueous medium [11].

Glutaraldehyde crosslinked film was more stable in water and remained insoluble in water. Swelling and de-swelling were done for all types of hydrogels. It was observed that hydrogels swelled up to 90% within 30 minutes, attained equilibrium within 1 hrs and retained its structure for 4 hrs. From the swelling and de-swelling results it can be inferred that these gels do not show much changes when swelled and de-swelled repeatedly [19].

Gel fraction per cent of obtained hydrogel was above 50% indicates that the hydrogel is mechanically stable with good gel strength. Glutaraldehyde crosslinking is responsible for the strength of the gel. The crosslinked κ-carrageenan-gelatin hydrogel is resistant to dissolution in water and improve the gel fraction percent. Compared to the non-crosslinked hydrogel, crosslinked hydrogel was more stable in water and remained insoluble in water [3, 11].

In the present study swelling characteristics of the hydrogel was performed by measuring swelling ratio, water absorption per cent and equilibrium water content. The maximum swelling index was in the range of 1600-2200 percent, water absorption percentage ranges 1500-2100 percent and maximum equilibrium water content of 94%. These values are high enough to prevent the wound bed from exudate accumulation [17].

Wound exudate is produced as a normal part of healing process prevent the wound bed from drying out. It consists of fluid that has leaked out of blood vessels and the excessive secretion may delay wound healing. Because of the water absorption property of hydrogel material wound exudate accumulation is highly reduced and provide a moist environment for promoting wound healing [20].

Wounds provide a moist, warm and nutritious environment that is conducive for microbial colonization and proliferation. Infections from the external environment retard the rate of wound healing significantly. So wound dressing should protect the wound from penetration of pathogenic microorganisms from the external environment to surface. In the present study *In-vitro* microbial penetration assay showed that, there were no bacteria passing through the hydrogel during day-by-day observation for 30 days, indicate that cross-linked hydrogel film was a potential barrier for microbes [12],[15].

4. Conclusions

From the current study it was found out that glutaraldehyde crosslinking of κ-carrageenan-gelatin hydrogel improves the physical characteristics by crosslinking the monomeric units. The physical characterization of hydrogel revealed that gel fraction (%) was 53.99 ± 0.14%, swelling ratio (%) was 1801.385 ± 94.31%, water absorption(%) was 1701.385 ± 94.31% and equilibrium water content (%) was 94.38 ± 0.27%. It was also found that the gel do not permit bacteria to penetrate though it

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6. Conflict of Interest

The authors declare that there are no existing conflicts of interests with respect to this research work.

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