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RESEARCH ARTICLE PHARMACEUTICS

IN VITRO EVALUATION OF TOPICAL GEL PREPARED USING SILK FIBROIN AT DIFFERENT CONCENTRATION OF GEL ACCELERATING AGENT-GLYCEROL

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ABSTRACT

The pharmaceutical utility of silk fibroin as a possible material for wound healing was investigated. Topical Silk fibroin gel (SFG) was prepared from its aqueous solution. The rate of gelation was sufficiently accelerated by addition of glycerol to silk fibroin (SF) aqueous solution. Rheological properties of topical SFG were evaluated using controlled stress rheometer. The glycerol content affected the spreadability, extrudability, bioadhesivity, linear viscoelastic region (LVR), tan δ, and creep recovery of topical SFG. It was found that topical SFG was able to prepare at room temperature (20 ± 5°C).

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KEYWORDS

Topical gel, Gel accelerating agent- glycerol, Bioadhesion, LVR, Creep recovery

INTRODUCTION

Silk fibroin (SF) is a natural protein produced by the domestic silkworm, *Bombyx mori.* Recently silk fibroin (SF) has been used in various research field such as the medical, biomaterial, and food additives fields because of its unique physicochemical properties and it's useful to humans[1,2]. The silk fibroin fulfills the principle requirements for activating epidermal cells of greatly improved cellular adhesion rate, multiplication, intercellular adhesion and growth of cell layers. It act as a wound healing material has biocompatibility and infection controllability as essential properties required for such a material, especially excellent flexibility and water absorption properties, thereby accelerating smooth regeneration of a skin defect[3,4].

The effects of a silk film on full thickness skin wounds of mice found that wounds dressed with sterilized silk film healed 7 Days faster than those covered with traditional dressing. The silk film also enhanced collagen synthesis, reduces oedema & scarring due to inflammatory responses & promoted epithelialization[5]. The silk fibroin films displayed several useful properties expected of soft-tissue compatible materials, for example good mechanical strength in the wet state, resistance against enzymatic degradation, ability to attach cells and promote the growth of the attached-cells, resistance to g-ray radiation and so on. Therefore silk fibroin is excellent additives for cell culture, wound healing promoters and cosmetic compositions[6]. By knowing such useful application of this natural protein it may be use for wound healing purpose in a suitable and stable dosage form like topical gel.

For the preparation of topical gel, gel forming agents like Carboxymethyl cellulose, Methylcellulose, Hydroxypropyl cellulose, Carbomer, Poloxamer, Polyacrylamide, Besitoinite etc. are essential[7]. These polymers give the structural network, which is essential for the preparation of gels. These gel bases give only applicable rheological properties to the gel but not wound healing activity in wound healing gel formulations. In future if it is possible to formulate wound healing topical gel in which not only drug but also gel base gives activity then it will be synergistic effect due to both drug and gel base. Silk fibroin protein may be a good candidate for this. Addition of glycerol in silk fibroin aqueous solution may bring polypeptide chains very close to form the β - structure in a short time[2] and give gelation of the protein.

In our present study, we prepared topical gels from same amount of the silk fibroin protein by using different concentrations of glycerol as gel accelerating agent and evaluate by means of protein content, gelation time, physical appearance, spreadability, extrudability, bioadhesivity and rheological measurements of that respective formulations.

MATERIALS AND METHODS

Silk cocoon purchased from Sericulture Institute and Training Centre, Pune. Cellulose tubing (Sigma) with molecular cut-off 12,000-14,000 Da were used for dialysis. Lithium Bromide, Glycerol, Sodium carbonate, Petroleum Ether (40-60), Citric acid (Merck Ltd., Mumbai, India).

(1) Preparation of silk fibroin aqueous solution[8,9]

The cocoon was cut into small pieces and washed with distilled water. It was then degummed by treatment with 0.5 wt% Na_2CO_3 solution at 98-100 ˚C for 1 hr. and

washed with hot distilled water till the washing were free from alkali. After air drying, fibers were defatted by 24 hrs. treatment with Petroleum ether (40-60) and were dried again. The degummed fibroin (5g) was dissolved in 15 ml 9 M LiBr solution with continuous stirring at 48-50 ˚C. The resulting fibroin solution was filtered and dialyzed in a cellulose tube against distilled water for 3 days. During this period distilled water was replaced with a fresh one after every 2-3 hrs. interval. The obtained 20 ml fibroin solution after dialyzed was centrifuged at 4000 rpm for 20 min. (Research centrifuge R24, Remi Instruments, India) and filtered through muslin cloth. Same like this, two more batches were prepared.

(2) Total protein content of silk fibroin solution

Total protein in the solution was calculated by Biuret method. Mix all required reagents well as shown in **table 1** and keep in water bath at 37 ˚C for 10 min. Measure the optical density of test (T) and Std (S) at 555 nm by UV spectrophotometer (Jasco V 530, Jasco, Japan).

Table 1.
Procedure for biuret testing

Pipette out into test tube	Test (T)	Std (S)	Blank (B)
Silk fibroin solution (ml)	0.05	-	-
Protein Std.-1 (ml)	-	0.05	-
Biuret Reagent-3 (ml)	2.5	2.5	2.5

Calculations

$$\text{Total protein in g/100 ml} = \frac{(T)}{(S)} \times \text{Conc. of total protein i.e. 6.9 g/100 ml}$$

(3) Preparation of topical SFG

Three different batches of silk fibroin aqueous solution containing 5 g of silk fibroin in 20 ml of each were prepared. To these batches, selected concentrations of glycerol and methyl paraben were added and final volume adjusted to 100 ml by distilled water at room temperature (20 ± 5˚C). The pH of the mixture was adjusted to 3.00 or 4.00 with 1M citric acid[2].

Table2.
Formulation design for preparation of topical SFG

Content	Batch A	Batch B	Batch C
Silk fibroin	5 g	5 g	5 g
Glycerol	60 ml	70 ml	80 ml
Methyl paraben	15 mg	15 mg	15 mg
Distilled water	upto 100 ml	upto 100 ml	upto 100 ml

(4) Determination of gelation time[2]

The time required for the formation of a gel from solution was determined according to the procedure of Okenfull et al., 1986. A fixed weight silk fibroin solution was placed in a flat-bottomed cylindrical beaker and was tilted at definite time intervals, the time required to form a gel just strong enough to retain its shape in position was recorded.

(5) Spreadability study of topical gel

Spreadability was determined by apparatus suggested by *Mutimer et al* [10] which was suitably modified in the laboratory and used for the study. It consists of a wooden block, which was provided by a pulley at one end. By this method, spreadability was measured on the basis of 'Slip' and 'Drag' characteristics of gels[11]. A ground glass slide was fixed on this block. An excess of gel (about 2 g) under study was placed on this ground slide. The gel was then sandwiched between this slide and another glass slide having the dimension of fixed ground slide and provided with the hook. A 1 Kg weight was placed on the top of the two slides for 5 minutes to expel air and to provide a uniform film of the gel between the slides. Excess of the gel was scrapped off from the edges. The top plate was then subjected to pull of 80 g. With the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5 cm be noted. A shorter interval indicates better spreadability[12,13]. Spreadability was then calculated using the following formula:

$$S = M \times L / T$$

Where, S = is the spreadability, M = is the weight in the pan (tied to the upper slide), L = is the length moved by the glass slide and T = represents the time taken to separate the slide completely from each other.

(6) Extrudability study of topical SFG[14]

In the present study, the method adopted for evaluating gel formulation for extrudability was based upon the quantity in percentage of gel and gel extruded from lacquered aluminum collapsible tube on application of weight in grams required to extrude at least 0.5 cm ribbon of gel in 10 seconds. More quantity extruded better was extrudability. The measurement of extrudability of each formulation was in triplicate and the average values are presented. The extrudability was than calculated by using the following formula:

Extrudability = Applied weight to extrude gel from tube (in gm) / Area (in cm2)

(7) Ex-vivo bioadhesive strength measurement of topical SFG

A modified balance method was used for determining the *ex-vivo* bioadhesive strength[15]. Fresh goat hairless skin was obtained from a local slaughter – house and used within 2 hours of slaughter. The skin was separated by removing the underlying fat and loose tissues. The membrane was washed with distilled water and then with 0.1 N NaoH [16,17]. The modified *Patel et al (*2007) method was used for the measurement of bioadhesive strength[16]. The fresh skin was cut into pieces and washed with 0.1 N NaoH. Two pieces of skin were tied to the two glass slide separately from that one glass slide was fixed on the wooden piece and other piece was tied with the balance on right hand side. The right and left pans were balanced by adding extra weight on the left hand pan. 1 g of topical gel was placed between these two slides containing hairless skin pieces, and extra weight from the left pan was removed to sandwich the two pieces of skin and some pressure was applied to remove the presence of air. The balance was kept in this position for 5 minutes. Weight was

added slowly at 200 mg/ min to the left – hand pan until the patch detached from the skin surface. The weight (gram force) required to detach the gel from the skin surface gave the measure of bioadhesive strength[15,17]. The bioadhesive strength was calculated by using following:

Bioadhesive Strength = Weight required (in gms) / Area (cm_2)

(8) Rheological measurements

For investigation of rheological properties of silk fibroin gel, a controlled stress rheometer was used (Viscotech Rheometer, Rheologica Instruments AB, Lund, Sweden). Data analysis was done using Stress Rheologic Basic software, version 5.0. A cone and plate geometry was used with 25 mm diameter and cone of 1.0 °. The gel was pressed between cone and plate with a gap of 0.5 mm and all measurements were carried out at 25°C. To determine the viscoelastic properties of silk fibroin gel, following tests were performed.

(8.1) ***Oscillation stress sweep***

The samples were exposed to increasing stress (0.1-200 Pa) at a constant frequency (0.1 Hz). The ability of gel to resist the deformation with applied stress was recorded in terms of trends of elastic modulus (G′), viscous modulus (G″), and phase degree with applied stress.

(8.2) ***Oscillation frequency sweep***

The samples were exposed to a stepwise of increasing frequency (0.1-100 Hz) at a constant stress in the field of linear viscoelastic region (LVR). G′, G″, phase degree and tan δ were recorded against frequency.

(8.3) ***Creep-recovery***

The test was carried out at stress in LVR, which was maintained constant for 100s. It was then instantly removed and the recovery was followed for 200s. The Creep compliance J (1/Pa) (defined as the ratio between the measured strain and the applied stress) is monitored against time. The creep-recovery percent can be calculated with the following equation:

$$\delta J = [\frac{J(100s) - J(300s)}{J(100s)}] \times 100$$

RESULTS AND DISCUSSION

All prepared topical gel formulations contain different amount of glycerol showed following results of protein contain, gelation time, spreadability, extrudability, bioadhesivity and different rheological properties.

(1) Total protein content

A Biuret reagent contains copper sulphate. Copper ion reacts with peptide molecule and produce violet colorization. This can be quantitified using colorimetric analysis. Silk fibroin in silk fibroin solution had presented λmax at 555 nm. The final protein content of Batch A, Batch B and Batch C was 4.9894 g (99.78%), 4.9898 g (99.80%), 4.9895 g (99.77%) respectively.

(2) Gelation of SF aqueous solution

The gelation time of SF aqueous solution contained 5 g of silk fibroin without addition of any gelation rate accelerating agent like glycerol, methyl alcohol, PEG, sulfuric acid, glutaraldehyde was 24-36 hrs. The gelation of SF took place while its acidic solution stood, and was due to the transition from the random coil to the β-structure by intermolecular hydrogen bonds between its polypeptide chains. The gelation time depends on silk fibroin, glycerol content and pH conditions. When pH of the mixture was

less than 2.5 or more than 4.5 gelation was not observed. Addition of glycerol to proteins caused the spherical exclusion of glycerol from the protein domain and also caused preferential hydration and stabilization of the protein and thus formation of the gel. From these considerations, in the case of the topical SFG prepared with high glycerol content, polypeptide chains may be brought very close to form the β- structure in a short time. The gelation time for Batch A, Batch B and Batch C was 5 min, 3 min and 2 min. respectively. The gelation time is sufficiently decreased with an increase in glycerol content. This apparently indicates that the portion of polypeptide chain which forms the β- structure increased. To, summarize this study; the gelation time of topical SFG depended upon the glycerol content[2]. It may be proposed that there may be H-bonding between –OH group of glycerol and H-group of silk fibroin leading to formation of stable gel structure. With increase in glycerol concentration, free water was displaced leading to the increased H-bonding between free water and glycerol; forming a weak gel structure[18]. Viscoelastic gelation for topical purpose with applicable properties like spreadability, extrudabillity and bioadhesive was not observed below the 60% v/v of glycerol content in 5% w/v of silk fibroin solution with water. Therefore we prepared three batches with 60% v/v, 70% v/v, and 80% v/v of glycerol in 5% silk fibroin solution with water. These gels were clear or transparent in appearance.

Table 2

Spreadability study, Extrudabillity study and Bioadhesive strength results of topical SFG

Topical SFG	Spreadability (gm.cm/sec.)	Extrudability (gm/cm^2)	Bioadhesivity strength (gm/cm^2)
Batch A	15.81	18	1.89
Batch B	15.85	18.50	1.30
Batch C	16.0	18.79	1.12

(3) Spreadability, Extrudabillity and Bioadhesive strength results of topical SFG

From obtained results we found that topical SFG having greater spreadability, extrudabillity and bioadhesive strength. But Out off three batches Batch A results were more acceptable because of good bioadhesivity and sufficient spreadability and extrudabillity. **Table 2** shows that spreadability and extrudability of Batch B and C was more than Batch A due to more amount of glycerol might have immobilized free water, necessary for junction zone formation and destabilized the gel system[2]. Therefore from above results it was conclude that Batch A is more stable and viscoelastic one.

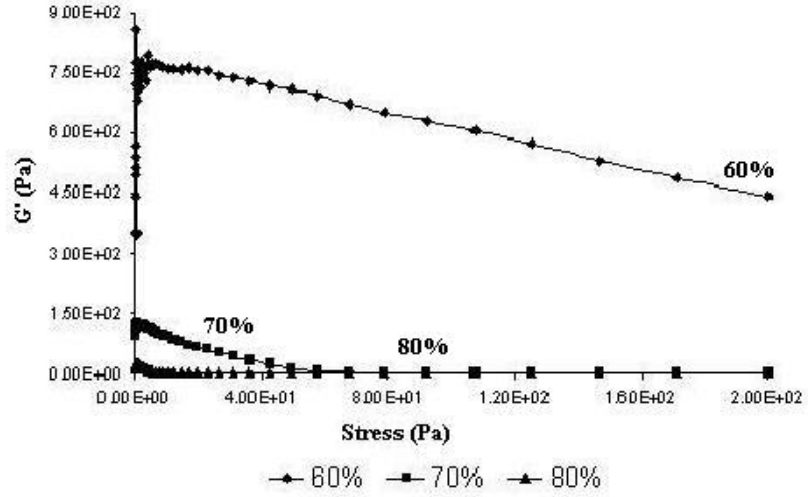
(4) Rheological studies of topical silk fibrin gel

Rheological studies can be performed by static (Rotational) and dynamic (Oscillatory) measurement mode. The dynamic rheology provides a more direct correlation with microstructure than steady rheology since the materials can be examined in their at-rest state without causing any disruption of their underlying structures[19]. Effect of addition of the other excipients on the gel properties and its performance can be predicted from systematic rheological studies. The concentration of glycerol affect on rheological properties which indirectly correlated to the gel characteristics such as consistency, clarity, appearance, spreadibility, extrudability and bioadhesivity.

(4.1) Oscillation stress sweep

In an oscillation stress sweep test the response of the material (strain) is measured while exposing the material to an increasing stress and a constant frequency. In the linear viscoelastic area the ratio of stress and strain is a function of time alone[20]. The solid moduli (G') represent the total energy stored in the elastic bonds of system while viscous moduli (G'') present total energy expelled from system. Stress sweep was carried out to determine the linear viscoelastic region (LVR), where G' is independent of applied stress. LVR has great significance for frequency sweep structure as we have to sure that the frequency sweep is carried out in ground state of the material i.e. there must not be any breakdown of the structure. The G' values for silk fibroin gel prepared with different glycerol concentration are given in **fig. 1**.

Fig. 1
Stress sweep for G'



Gel with 60% v/v of glycerol had shown maximum G' and LVR as compared to other system and thus it was comparatively more stable over applied stress range. Addition of the glycerol to silk fibroin solution might have brought the H-bonding between the silk fibroin and –OH of glycerol leading to formation of junction zone ultimately forming gel structure. In oscillatory stress sweep test, system with higher LVR and G' are considered as more elastic[21]. However, addition of higher amount of the glycerol might have immobilized free water, necessary for junction zone formation and destabilized the gel system. Therefore both 70% v/v and 80% v/v glycerol containing gels presented lower G' and LVR over applied stress indicating formation of weak gel compare to first one.

Fig. 2, 3 and 4 represent the behavior of G' and G'' over applied stress for silk fibroin gel prepared from 60% v/v, 70%v/v and 80% v/v glycerol, respectively.

Fig. 2
Stress sweep for G' and G'' of 60% glycerol contained gel

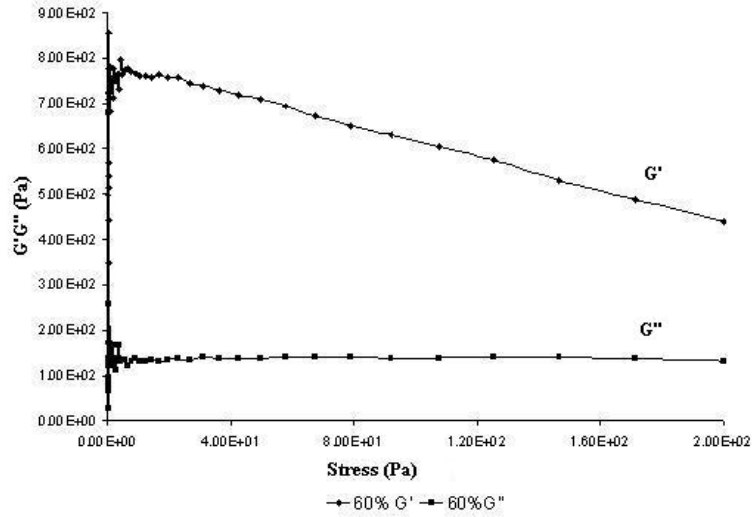


Fig. 3
Stress sweep for G' and G'' of 70% glycerol contained gel

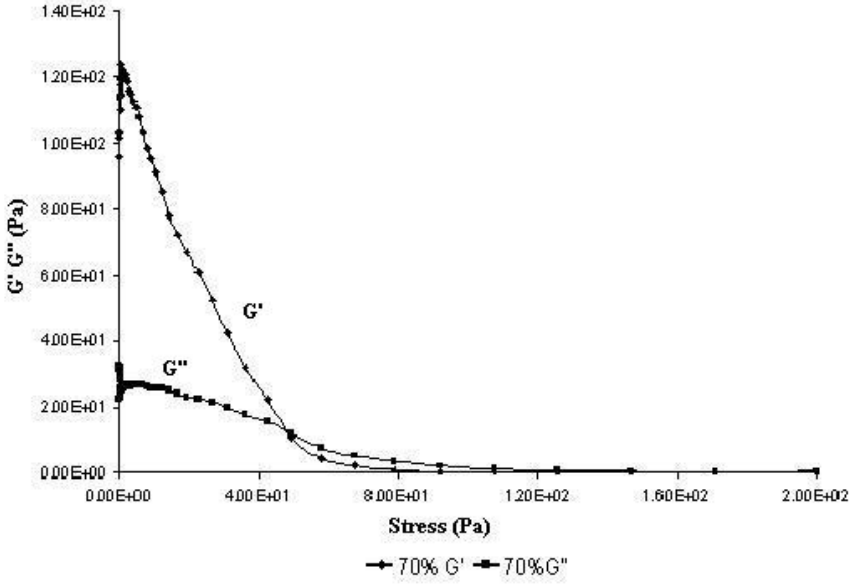
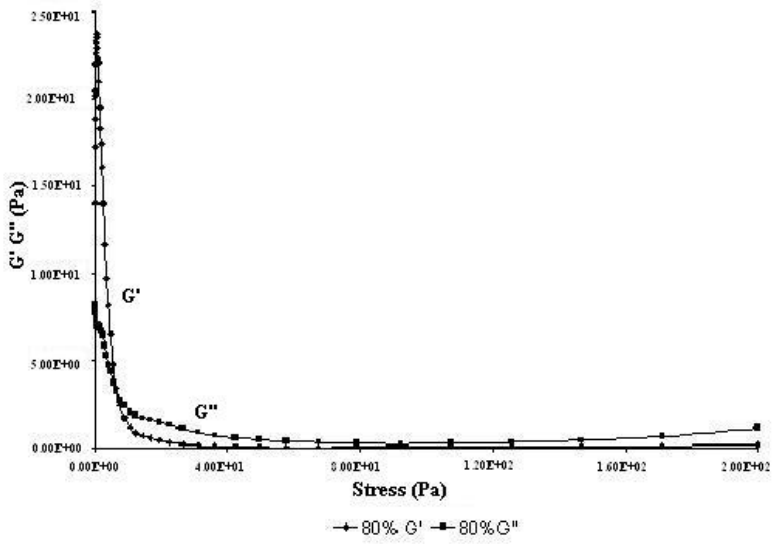


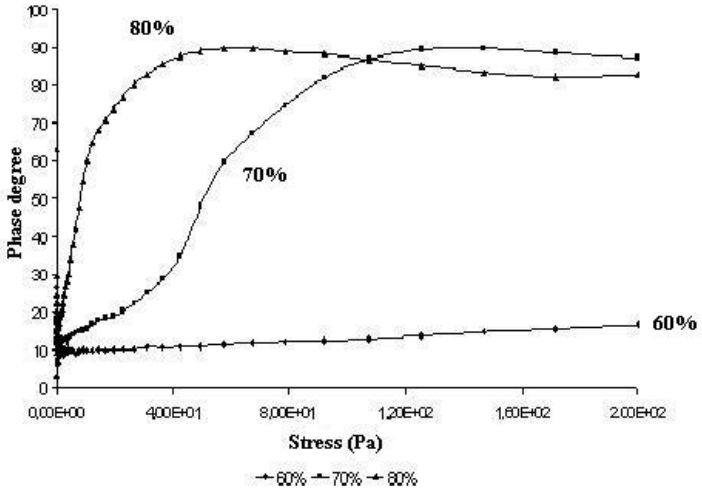
Fig. 4
Stress sweep for G' and G'' of 80% glycerol contained gel



The figures clearly indicated that for 60% system, G' was greater than G'' over all applied stress, while for 70 and 80% system, G' was higher than G'' at lower stress but at higher stress G' was lower than G''. Thorgeirsdottira, et al. mentioned that trend of G'>G'' is for gel and solid like materials[22].

The phase angle is good indicator of viscoelastic nature of system, being measure of the lag in sine response after an oscillatory stress has been applied to the sample. For perfectly elastic system, phase degree must be close to 0° and for viscous system it must be close to 90° [23]. The effect of stress on phase degree is given in **fig. 5.**

Fig. 5
Stress sweep for phase degree



There was a phase shift observed for gel with higher glycerol content (70 & 80 % v/v glycerol) with respect to applied stress. These systems have presented the change of behavior from elastic to viscous one, thus proving the instability of the system. Therefore, from stress sweep, it was concluded that silk fibroin prepared with 60% v/v glycerol are more stable and elastic one.

(4.2) Oscillation frequency sweep

Generally, frequency sweep measurements give good information on microstructures of the gel system. In this the degree of dependency of G' and G'' is examined over the applied frequency range.

Fig. 6
Frequency sweep for G'

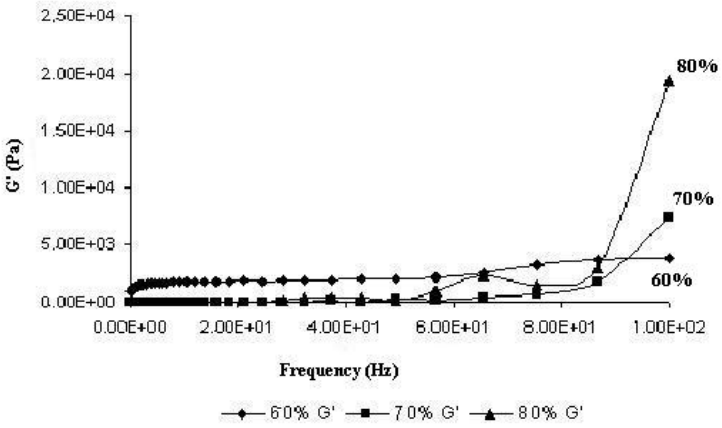


Fig. 6 presets the behavior of G' over the applied frequency range. From figure it was concluded that 60% v/v glycerol containing gel had shown independent behavior of G' over applied frequency, while 70% v/v and 80% v/v system have exhibited a monotonous increase in solid component. It is possible that monotonic increase in G' at higher frequencies means the partial breakage of the interconnected network, inferred from the existence of the plateau region at lower frequencies, which represents a true cross-linked polymer gel network. It is, therefore, concluded that the gel network is retained at low frequencies and, on the other hand, destroyed by the more frequent changes of the displacement at higher frequencies due to the formation of too rigid and brittle structures[24].

Trend of viscous moduli and phase degree over applied frequency are shown in **fig. 7 and 8,** respectively.

Fig. 7
Frequency sweep for G''

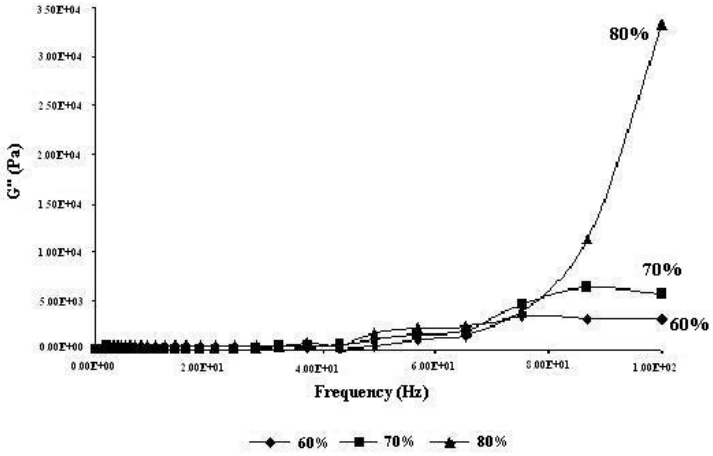
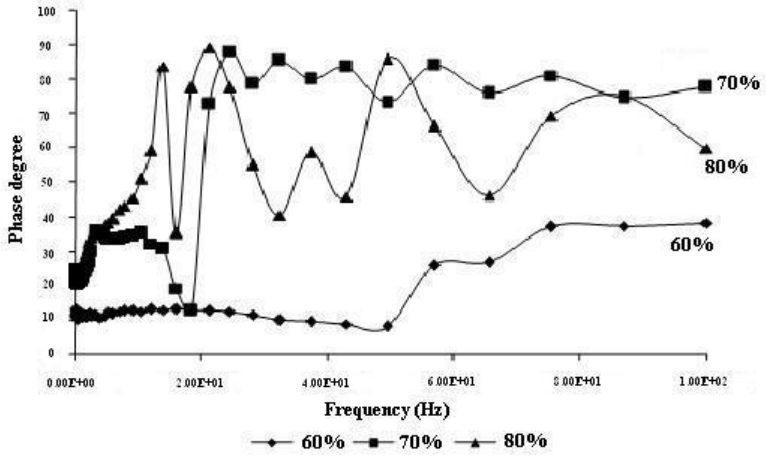


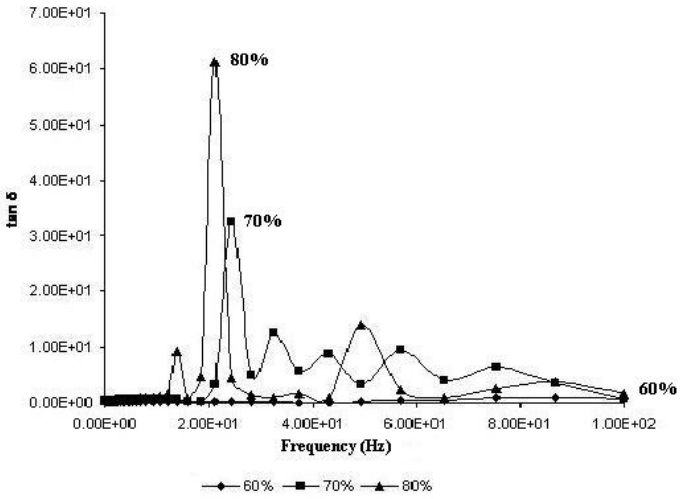
Fig. 8
Frequency sweep for phase degree



It was clear that 60% v/v glycerol containing system had shown low dependency on frequency for viscous moduli and phase degree than 70% and 80% system. The value of phase degree for 60% v/v glycerol containing system was well within viscoelastic region. The increase in G'' and phase degree clearly indicated the weak structure of 70 % v/v and 80% v/v glycerol containing systems.

In frequency sweep test a new terminology was introduced i. e. Loss tangent (tan δ). It is the ratio of loss modulus (G'') and storage modulus (G'). The higher the loss tangent less is the elastic behavior[20]. System with values of tan δ <1 characterize a predominant elastic behavior and values of tan δ >1 indicate a prevailing viscous behavior[21]. For 60%v/v glycerol containing SFG system tan δ was clearly below 1, while for 70% and 80% system it was much higher than 1 **(Fig. 9).**

Fig. 9
Frequency sweep for tan δ



With this prospective, only 60% system is elastic and stable one. In frequency sweep, there must be non-dependency of both G' and G'' over the applied frequency and condition of G'>G'' must be prevalent over all rage[25]. Considering these criteria, 60% v/v glycerol containing SFG was a gained emerged as stable and elastic one.

In stress sweep the difference between the systems was wide as compared to that of frequency sweep. Generally, frequency sweep measurements give good information on microstructures of the gel system. However, in this study it was found that the frequency sweep test was not enough to differentiate the structure changes from string-like entangled structure to honeycomb-like rigid structure. The frequency sweep test cannot
detect this transformation since the measurement is done within linear response ranges, which mean no structure breakage during measurement. On the other hand, the stress sweep test may induce destructive effect on the network system, especially, at higher shear stress region. Rigid structures such as honeycomb structure in this study can contribute to upward shift of stress sweep curve.

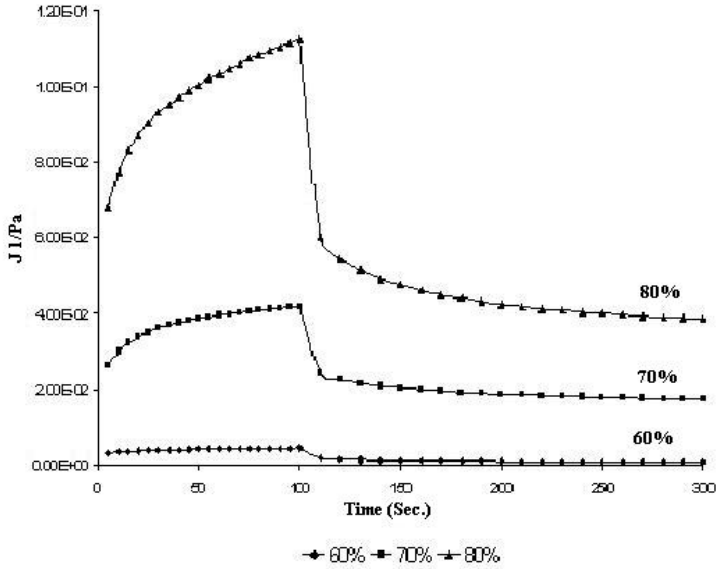
(4.3) Creep recovery test

In the creep test a constant stress within LVR is applied for a fixed time (100S) and then removed (200S). In this the strain of a sample is determined as a function of time. During the creep test the strain is measured as a function of applied stress and presented in terms of compliance, J.

The creep-recovery percent for 60% v/v, 70% v/v and 80% v/v glycerol contained topical SFG was 85.48, 58.44 and 65.79%, respectively. As the 60% system has highest elastic recovery, it is said to highly stable and easy to process, store and apply system.

The results of creep recovery test are given in **fig. 10.**

Fig. 10
Creep recovery test for prepared silk fibroin gels



From graph, it was clearly seen that 60% system had lowest compliance value (J). Lower the compliance, higher is elasticity[26]. Therefore, from creep recovery test, it was inferred that all system have shown considerable elastic and viscoelastic recovery but, silk fibroin gel with 60% v/v glycerol had presented highest recovery.

From oscillatory rheological measurement, it was observed that topical silk fibroin gel of 5% fibroin prepared from 60% v/v glycerol was highly viscoelastic and stable. It was having high recovery capacity also. From these rheological properties, it can be claimed that this system having good spreadability, extrdability, and applicability.

CONCLUSION

From this study we can conclude that proper concentration of silk fibroin and gel accelerating agent form applicable and stable topical silk fibroin gel. As per activity need we can change the concentration of silk fibroin from minimum to maximum, but by changing this concentration there must be change in percentage of gel accelerating agent also for the required topical gel properties. As noted in introduction, we can formulate topical silk fibroin gel with synergistic activity by incorporating any wound healing agent at silk fibroin aqueous solution step. The percentage of drug, silk fibroin, glycerol and pH of the silk fibroin solution affect gelation time, micro structural networking and ultimately those rheological properties of the gel.

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