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Preparation of non-toxic regenerated silk fibroin hydrogel by using gluteraldehyde as cross linking agent

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Abstract:

In present years' researchers are interested in the fabrication of non-toxic hydrogels which can benefit the needs of mankind. Hydrogels are three dimensional structures which absorbs large quantity of water in it and which also imitates the ECM when injected into the body.

In order to achieve that we fabricated a non-toxic silk fibroin hydrogel by cross-linking with glutaraldehyde. The biophysical characterization of the fabricated GARSF (Gluteraldehyde regenerated silk fibroin) hydrogel was done by SEM, FTIR-ATR. Swelling behavior, degradation studies and cytotoxicity studies suggests that the fabricated GARSF hydrogels as a good material for the cell growth and proliferation.

Keywords *A. mylitta*, Regenerated silk fibroin, Gluteraldehyde, cytotoxicity

I. Introduction:

The silk fibroin of *B. mori* is abundantly studied or utilized from the past many years but wild silks *A. mylitta* was unexplored in the field of biomaterials. Silkworms cocoons of *A. mylitta* contains large amount of silk fibroin in it. Silk fibroin of *A. mylitta* has many magnificent qualities like biodegradability, biocompatibility, strong mechanical strength, because of which SF is widely used in various fields such as tissue engineering in different shapes like scaffolds, films, sponges, hydrogels, etc (1, 2 and 3). SF has a significant benefit when compared to many other materials is that easily soluble in water and is sensitive under temperate conditions, making it a fascinating material for drug delivery systems and sensitive bioactive agents.

To fabricate any biomaterial for biomedical applications, it should have strong mechanical strength and morphology which serves as drug delivery system or any other tissue engineering applications (4). To build such a biomaterial which contains all necessary requirements for both implants and drug delivery is a challenging process in regenerative repair. To modify the structure and properties of SF, numerous methods have been employed involving mixing with synthetic polymer or chemical alterations to tackle the wide range of biomedical needs (5, 6). The most frequently used method to enhance the mechanical strength and its stability in aqueous phase, is cross-linking technique. Till today SF is only used for textiles and some applications of tissue engineering through physical method by altering

pH, temperature etc., chemical cross-linking is least explored. However, only small amount is known about the influence of cross-linking and cross-linking agents on the properties of SF matrix (7, 8, and 9).

Cross linking is the method used to overcome the drawbacks of the biomaterials (10, 11). Apart from linking molecules and boosting molecular weight, crosslinkers also often provide enhanced mechanical properties and stability. However, crosslinking also alters the rheology of the polymers, which causes processing challenges and a possible increase in cytotoxicity. It also decreases degradability, reduces the availability of functional groups in the crosslinked polymer, and decreases availability of functional groups in the crosslinked polymer (12). Numerous crosslinkers and crosslinking strategies are employed based on the type of biopolymer to be crosslinked and the degree of desired property improvement (13, 14, and 15).

Most of the chemical croslinkers employed, GA is largely utilized because of its reaction with protein and carbohydrate's functional groups, which gives considerable increase in the tensile strength of the protein (16, 17). Even though glutaraldehyde significantly improves mechanical behavior, conflicting information has been presented regarding the cytotoxicity of materials that have been crosslinked with glutaraldehyde (16).

Glutaraldehyde (GA) is frequently employed as a fixative for biological tissues (7) as well as a general coupling agent for molecules and polymers containing amines. This method of creating cross-linked SF films with GA has improved their mechanical properties (9). However, the concentration used determines the extent to which glutaraldehyde is cytotoxic; it has been evidenced that up to 8% of the compound is not toxic (16).

To fabricate nontoxic hydrogel by crosslinking technique, we used regenerated silk fibroin with 3 % gluteraldehyde as a crosslinker. The SEM analysis of the fabricated hydrogel revealed the microporous structures which aids in the providing the favorable conditions to the cell growth. FTIR ATR results showed the crystalline structures of the GARSF hydrogel.

II. Materials and methods:

Sigma Aldrich provided all compounds, including Gluteraldehyde, calcium nitrate tetra hydrate $Ca(NO_3)_2.4H_2O$, sodium carbonate (Na_2CO_3) and protease XIV. The Regional Tasar Research Station in Warangal, Telangana, India provided cocoons of Wild silkworms *Antheraea mylitta*. Silk fibroin protein was isolated from the cocoons by using procedure described previously (18).

2.1. Preparation of Silk Fibroin Solution:

The degummed silk was washed numerous times in deionized water to eliminate salts after the degumming procedure. To eliminate water, the degummed silk was dried overnight at 40°C in a hot air oven. 1.0 g degummed silk was added to a 1:80 $Ca(NO_3)_2.4H_2O$ salt, agitated for 1 hour at 80°C, cooled, and suction filtered. The filtrate was subjected to dialysis by using pre-treated dialysis tubing against deionized water. After dialysis, the regenerated silk fibroin solution was refrigerated at 4°C and lyophilized for further use.

2.2. Preparation of GARSF Hydrogel:

A 3% gluteraldehyde solution was prepared, then adding silk fibroin suspension at 6% loadings and stirring for 1 hour at 95°C. Finally, the hydrogel was cast onto a glass tube with a precise level and dried for two days at room temperature.

2.3. Scanning Electron Microscope:

The gold was vacuum-coated onto the prepared GARSF hydrogel. A scanning electron microscope (Jeol J S M-I T 500) was used to examine the surface morphology of GARSF hydrogel, which was imaged at a voltage of 15 kV. The images were taken at the high resolution of 200µm.

2.4. Fourier Transform Infrared ATR Spectroscopy:

Infrared spectra of the gel samples were collected by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Tensor 27, Brucker Optics, Germany) under absorbance mode from 4000 - 400 cm-1 at 4 cm-1 scan resolution. The starting gluteraldehyde and regenerated silk fibroin powder was used as control. The hydrogel is cut into approximately small round shape.

2.5. Cytotoxicity Assay:

The biocompatibility of GARSF hydrogel was determined by evaluating cell viability in the NIH3T3 cell line using the MTT test according to the ISO 10993-5-9-2009 procedure. In living cells, 25 MTT dye combines with mitochondrial reductase to produce purple colored (E, Z)-5-(4, 5-dimethylthiazol-2-yl)-1, 3- dimethylformazan. $1x10^4$ cells were grown in a 24-well plate for 24 hours in DMEM with 10% FBS and 1% penicillin streptomycin for this experiment. GARSF hydrogel was combined with DMEM and incubated for 72 hours at 37^0 Celsius. To make GARSF hydrogel-DMEM extract, an aliquot of the medium was sterilized using a 0.22 m syringe filter (22). The cells' culture medium was replaced with a mixture of complete DMEM and GARSF hydrogel-treated media in an equal proportion. After24, 48, and 72 hours of GARSF hydrogel solution treatment, the medium was replaced to full DMEM. The cells were incubated for 4 hours with 15% MTT solution (stock solution 5 mg/mL) in PBS. Depending on the amount of viable cells, purple-colored formazan crystals produced. These purple crystals were dissolved in 200µL of methanol/DMSO (1:1), and absorbance was measured using a microplate reader at 595 nm (23).

2.6. Swelling Ratio:

Determination of swelling ratio of the fabricated GARSF hydrogel was done by cutting them into 1x1 cm and immersing them in 10 ml of PBS at 37^0C, pH=7.4. The soaked hydrogel was dried softly using tissue paper and weighed after 5, 10, 20, 30, 60 and 120 minutes. The swelling ratio was determined by using the formula

$$\text{Swelling ratio} = \frac{Ws - Wd}{Wd} X100$$

Where W_d id the weight of dry hydrogel and Ws is the weight of swollen hydrogel at interval times.

III. Results:

3.1. Surface morphology:

The morphology of the prepared GARSF hydrogel was observed by scanning electron microscope. The hydrogel showed micro porous structures of different sizes in the fig. 2 below. Addition of GA aids in network formation in the synthesized hydrogel which is beneficial for cell adhesion and cell proliferation.

Fig 1. GARSF hydrogel

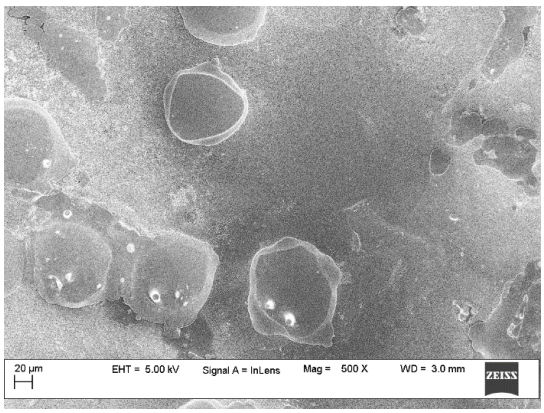

Fig 2. SEM micrograph of GARSF hydrogel

3.2. Fourier Transform Infrared ATR Spectroscopy:

The FT-IR spectra of RSF, GA and GARSF hydrogel was presented in the Fig 3. GA exhibited characteristic absorption band at 3336 cm^{-1} is corresponding to the hydroxyl (O–H) stretching vibration of aqueous solution of GA and band observed at 2963 and 1637 cm^{-1} are associated with C-H stretching of methylene ($–CH_2$) group and carbonyl (C=O) stretching vibrations, respectively (20). On other hand, structural confirmation of RSF was assessed by observing the amide absorption bands. The RSF showed the intense absorption band at 1635, 1537 and 1240 $cm^{-1}$are related to the stretching vibration of amide I, amide II, and amide III, respectively, that confirms the existence of β – sheet structure of silk fibroin (21). Further, the GA cross-linked RSF (GARSF) hydrogel had shown the similar FT–IR patterns to that of GA and RSF. However, the amide I and amide II absorption bands of RSF and carbonyl stretching band of GA are merged together and shifted to 1711 cm^{-1}. This large shift in the band position of amide I and amide II of RSF upon crosslink with GA was mainly due to the strong intermolecular interaction between the hydrogel components.

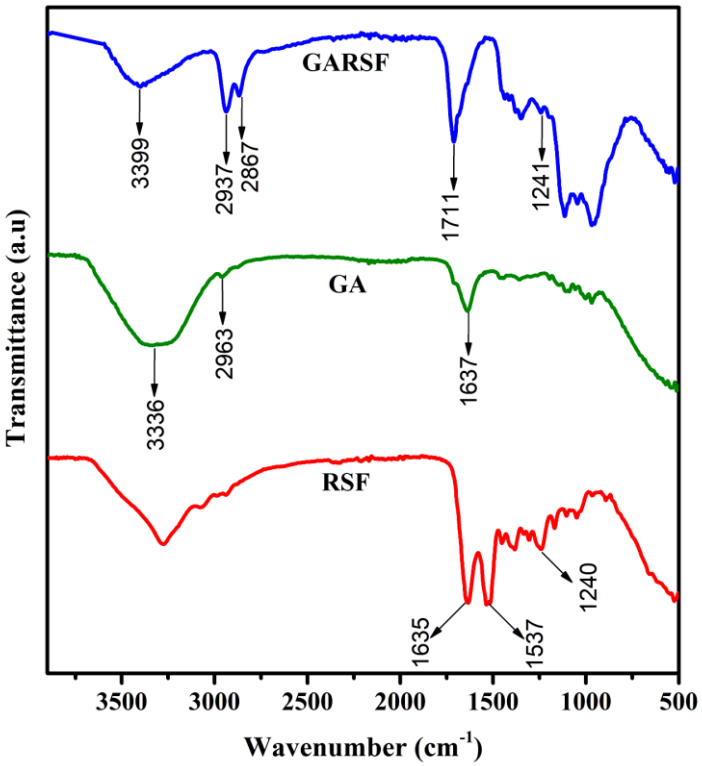

Fig 3. FT-IR ATR spectra of RSF, GA and GA/RSF Hydrogel

3.3. Cytotoxicity Test:

The cytotoxicity of the prepared GA cross linked RSF (GARSF) hydrogel was tested against L929 fibroblast cell lines. The hydrogels showed excellent cell viability and promoted cell growth in the experiment. These results showed that adding RSF to GA has a magnificent effect on the cell growth. Hydrogel showed cell viability of about 80% in the below fig 4.

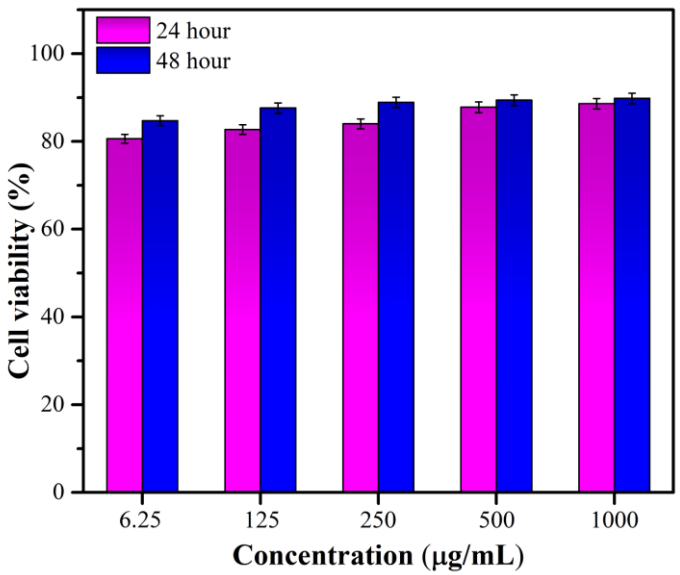

Fig 4. Cell viability of GARSF Hydrogel

3.4. Swelling Ratio:

The GARSF hydrogel showed maximum swelling ratio i.e. 199.92% at 120 minutes and after 120 minutes the swelling ratio remained constant showing the hydrogel is a good material for cell proliferation and cell adhesion providing favorable condition to the cells to grow.

Time (mins)	Swelling ratio (%)
	GARSF
5	169.67±0.19
10	173.56±0.20
20	181.32±0.29
30	192.88±0.40
60	195.15±0.42
120	199.92±0.09

Table 1. Percentage of swelling percentage of GARSF hydrogel against simulated degradation on Day 3, Day 5, and Day 7 of incubation in PBS. Values are expressed as mean ± SD of n = 3 in each group.

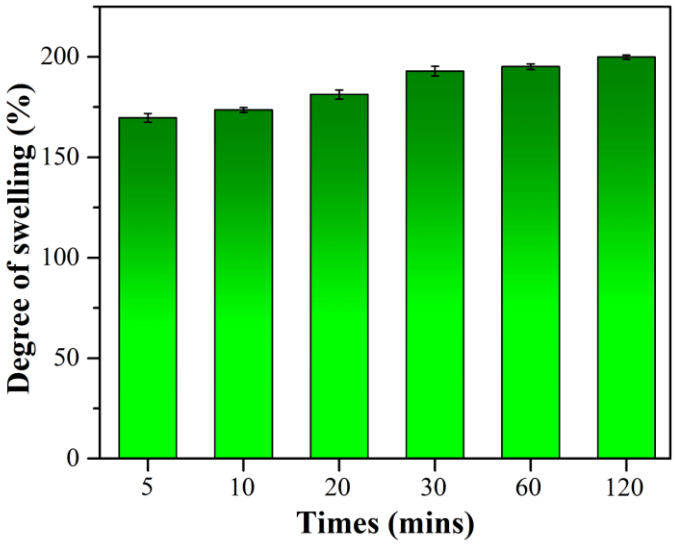

Fig 5. Swelling % of GARSF hydrogel

IV. Discussion:

A scanning electron microscope was used to examine the morphology of the prepared GARSF hydrogel with micro porosity structures of varying sizes. The addition of GA aids in the development of networks in the produced hydrogel, which is favorable for cell adhesion and proliferation. The RSF revealed an intense absorption band at 1635, 1537, and 1240 cm^{-1} that is connected to the stretching vibration of amide I, amide II, and amide III, respectively, confirming the existence of the silk fibroin β-sheet structure (21). Furthermore, the GA cross-linked RSF (GARSF) hydrogel displayed similar FTIR patterns to GA and RSF. The amide I and amide II absorption bands of RSF, as well as the carbonyl stretching band of GA, are combined and moved to 1711 cm^{-1}.

The cytotoxicity studies demonstrated that combining RSF with GA has a huge influence on cell proliferation. The cell viability of hydrogel was approximately 80 percent. The GARSF hydrogel demonstrated a maximum swelling ratio of 199.92% at 120 minutes, and the swelling ratio remained constant after 120 minutes, indicating that the hydrogel is an excellent material for cell proliferation and cell adhesion, providing favorable conditions for cell growth.

V. Conclusion:

We prepared a GARSF hydrogels with high desirable and tunable features for biomedical applications. SEM analysis revealed the micro porous structures which helps in the cell adhesion and proliferation. FTIR-ATR confirmed the β – sheet structure in the fabricated GARSF hydrogel. We also demonstrated that, by varying the concentration of silk fibroin in the hydrogel, the structural, pore size and Compression mechanical properties of the resulting hydrogels could be tuned. It is expected that this type of hydrogel can be used in cell migration investigation are needed as biomedical materials.

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