Research Article

International Journal for Asian Contemporary Research (IJACR)

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Fabrication, Characterization and Antifungal Activity Studies of Silk Fibroin Hydrogel as a Potential Controlled Release of Fluconazole

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Article info

Received: 02 April, 2021
Accepted: 30 April, 2021
Published: 03 May, 2021
Available in online: 04 May, 2021

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Link to this article: http://www.ijacr.net/upload/ijacr/2021-11-1005.pdf

Abstract

Silk is a natural protein-based fiber derived from the *Bombyx mori* and certain insects. The research aim is to develop silk fibroin (SF) hydrogels loaded model drug fluconazole to obtain controlled release profile for better patient compliance. An eco-friendly technique was introduced to prepare fibroin hydrogel by treating three different organic solvent of ethanol, propanol, and glycerol by adding 2% (w/v) silk fibroin aqueous solution 37 ºC. The hydrogel having higher SF content showed more stability and slower degradation rate. Then, to prove the potential of SF hydrogel as carriers for drug delivery, fluconazole was absorbed in the hydrogel. All hydrogel released fluconazole in a controllable manner, possibly due to the hydrophobic interaction between fluconazole and crystalline domain of SF. The fibroin hydrogel was characterized by using Fourier Transform Infrared Spectroscopy (FT-IR), Scanning Electron Microscopy (SEM), Thermo gravimetrical Analysis (TGA), Differential scanning calorimetry (DSC). The encapsulation efficiency and release profile of fluconazole was studied by UV-VIS spectrometry. The surface morphology of the hydrogel was affected by the formulation conditions. Better encapsulation efficiency (85.5±1.67%, 73.4±1.76%, 71.6±1.35%, respectively) of fluconazole for F16, F17, F18 formulation were achieved when glycerol, propanol, ethanol was used in the formulation. The release profile showed an initial burst release for fluconazole then a controlled release for the next 20 hours. The antifungal activity of hydrogel incorporated drug showed a positive response against Aspergillus Niger pathogen. Therefore, silk fibroin hydrogels might be a good candidate for controlled topical delivery of fluconazole.

Keywords: Silk fibroin, controlled release, hydrogels, drug delivery**.**

Introduction

Silk is a protein-based natural fiber derived from *Bombyx mori* and some insects. Silk fibroin has attracted great attention due to its potential applications in tissue engineering, enzyme immobilization, and controlled release drug delivery (Su *et al.*, 2016; Mariana *et al.*, 2014). Fibroin could be processed into versatile forms such as nanoparticles (Safdari *et al.*, 2016), microspheres, powders, hydrogels (Ansary *et al.*, 2014) and films (Asary *et*

al., 2016). The unique properties of silk fibroin (SF) such as slow biodegradation, superior mechanical properties, favorable process ability in combination with biocompatibility (Ansary *et al.,* 2016). Fibroin polymer is an important feature for the storage of a drug delivery device. Gelation of SF solutions through enrichment in β-sheet content than α conformation (Sharma *et al.,* 2013). The releasing behavior of drugs from hydrogels partially depend on the degree of swelling, which in turn depends on its degree of crosslinking, the ionization of the network and its hydrophobic hydrophilic/ balance (Wang *et al.,* 2006).

The characteristics of silk fibroin hydrogel is a three-dimensional polymer formed as a result of the sol-gel transition of silk fibroin solution in an aqueous solution initiated in the presence of organic solvent (Sharma *et al.,* 2013). The gelation transition is increased and enhanced by increasing the concentration of the protein present, temperature (Chen *et al.,* 2012) and the addition of organic solvent (Ribeiro *et al*., 2014). Some researchers reported that silk fibroin hydrogels were prepared from the aqueous silk fibroin solution obtained from the β sheet structure (Wang *et al.,* 2006). The rate of gelation in the solution depends on the PH of the silk solution (Tanaka *et al.,* 2009).

Hydrogel drug delivery systems are engineered technologies that use hydrogel for the targeted delivery and controlled release of therapeutic agents. Several applications in pharmaceutical and medical technology are based on dispersions of particles in a fluid or gel phase (Ribeiro *et al.,* 2014). Here we report that silk fibroin (SF) hydrogels loaded with fluconazole to achieve controlled release profile of the drug for better patient compliance.

Materials and Method

Materials

Matured *Bombyx mori* silk cocoons were provided by the Bangladesh Sericulture and Training Institute, Rajshahi, Bangladesh. Lithium Bromide (Fisher Scientific Company, USA), Petroleum Ether, Sodium carbonate, Silver Nitrate (Thomas baker chemicals limited, Mumbai, India), Model drug (Fluconazole), were used in this study.

Silk purification

The silk cocoon shells were cut by a sharp knife and the insects were taken out from inside. Cleaned white in color of cocoons shells (5.00g) were degummed twice in boiling solution of 0.02M Na_2CO_3 for one hour in a beaker to remove the glue like sericin protein. The hot plate was set to 70°C and spinning at the speed of 175 rpm for two sessions for two hours each. After that, the fiber was finally washed three times with boiling water and dried in an oven at 65°C for 50 minutes. The collected sericin free fibroin sample was weighed and found 3.626g. The silk fibroin was kept in a desiccator 2 days for further use.

Preparation of silk fibroin (SF) solution

SF solution was prepared by dissolving dry silk fibers (2.00g) into 10 mL of 9.28 M LiBr solution. For total dissolution purpose, the magnetic stirrer was set to 80°C at the speed of 220 rpm with continuous stirring until clear solution. After that, the fibroin solution was transferred into cellophane dialyzing bag. The dialyzing bag was immersed into 200mL of distilled water in a 500mL beaker. The solution was dialyzed at least 3 days. After dialysis silk fibroin solution were centrifuged at 20,000rpm for 15 minutes and collected samples were freeze dried overnight. The dried sample was used further for hydrogel preparation.

Preparation of Silk Fibroin Hydrogels

Different formulation of silk fibroin hydrogels was prepared by treating a 4% (w/v) silk fibroin aqueous solution at 37°C by adding freshly prepared of 50% (v/v) of glycerol, or 50% (v/v) of propanol, or 50% (v/v) of ethanol respectively. The composition of hydrogel has shown in the (Table 1). The mixed solutions total (20 mL) were put on molds (diameter 3.5 cm) and placed on a thermostatic bath at 37°C, until gelation occurred. After that, the gel was taken out and washed gently with redistilled water (Figure1).

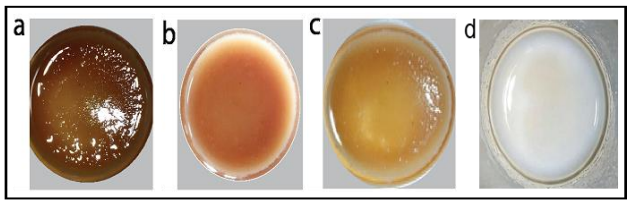

Figure 1. Silk fibroin hydrogels prepared in formulations: (a) F4, (b) F5, (c) F6 and drug loaded hydrogel in formulation of (d) F10

Table 1. Composition and gelation time of silk fibroin hydrogels prepared in different formulation.

Formulation No.	Vol. of silk fibroin solution (mL)	Vol. of glycerol solution (mL)	Vol. of propanol solution (mL)	Vol of ethanol solution (mL)	Vol. ratio of fibroin/solvent	Gelation time (hours)
F1	5	15	-	-	1:3	1
F2	5	-	15	-	1:3	2
F3	5	-	-	15	1:3	5
F4	10	10	-	-	1:1	3
F5	10	-	10	-	1:1	7
F6	10	-	-	10	1:1	12
F7	15	5	-	-	3:1	5
F8	15	-	5	-	3:1	12
F9	15	-	-	5	3:1	20

Incorporation of drug(fluconazole) into fibroin hydrogel

To encapsulate fluconazole into silk fibroin hydrogel, among all the formulation of fibroin solvent ration 1:1 showing better integrity where the following study was maintained. To evaluate loading efficiency of fluconazole in the fibroin hydrogel in which volume ration 1:1 also carried out. For the incorporation of fluconazole in silk fibroin hydrogels, (0.2g/mL) of 10 mL of fibroin dialyzed solution was added to the different formulation of fluconazole solution (0.01g/mL) prior to hydrogel formation. After that, 10mL of 50% (v/v) of glycerol, or propanol, or ethanol was added respectively in the formulations and the mixture was placed on molds of 4 cm in diameter and kept on a thermostatic bath at 37° C until gelation occurred. The gel was washed gently with redistilled water twice.

Characterizations

Encapsulation efficiency of fluconazole in silk fibroin hydrogel

Encapsulation efficiency of the drug in the fibroin hydrogel was calculated by UV-Vis spectroscopy (Table 2). Calibration curve for model drug (wave length 210 nm) was obtained by using five different concentrations of all stock solutions. Approximately 10mL of silk fibroin(0.2g/mL) dialyzed solution was suspended in 1 mL of fluconazole solution(.02g/mL) before gel formation. After that either 5mL of 50% (v/v) of glycerol, or 50% (v/v) of propanol, or 50% (v/v) of ethanol was added to the mixture prior to the gel formation respectively. After hydrogel formation the supernatant was taken out and analyzed for residual fluconazole concentration using UV-Vis spectroscopy (wave length 210 nm) (Chen *et al.*, 2012). Standard calibration curve of fluconazole solution was used for drug quantification. Amount of supernatant was used to calculate the amount of drug incorporated in silk fibroin hydrogel. All the experiment was performed in twice. Encapsulation efficiency was determined by the following equation,

Encapsulation Efficiency

$$(\text{w/w\%}) = \frac{amount\ of\ fluconazole\ in\ hydrogel}{fluconazole\ initially\ added} \times 100.$$

The results were calculated as mean ± standard deviation (S.D).

Table 2. Composition of silk fibroin loaded with fluconazole.

Formulation No.	Vol. of silk fibroin solution (mL)	Vol. of fluconazole solution (mL)	Vol. of glycerol solution (mL)	Vol. of propanol solution (mL)	Vol. of ethanol solution (mL)
F10	10	1	10	-	-
F11	10	1	-	10	-
F12	10	1	-	-	10
F13	10	2	10	-	-
F14	10	2	-	10	-
F15	10	2	-	-	10
F16	10	3	10	-	-
F17	10	3	-	10	-
F18	10	3	-	-	10
F19	10	4	10	-	-
F20	10	4	-	10	-
F21	10	4	-	-	10

In vitro released studies of drug from Silk Fibroin hydrogels

Silk fibroin hydrogels containing fluconazole were immersed in 50 mL of phosphate buffer solution (PBS) at p^H 7.4 followed by incubation at 37^0C 100 rpm with constant shaking. Samples of 2 mL of PBS were collected periodically, replaced with fresh PBS (p^H 7.4), and analyzed by UV-VIS spectrometry (wave length 210nm).

FTIR spectroscopy

Washed silk fibroin hydrogel as well as KBr were left to dry overnight in an oven at 45°C. Then, hydrogel and KBr were mixed up maintaining a ratio of 1:40. The samples were first cast on IR-transparent discs

and then placed under IMV-4000 multi-channel FTIR spectroscopy (Jasco FT/IR-6200, Japan). Analysis was performed in transmission mode using the microscope in Central Science Lab, Rajshahi University. The spectra were analyzed over a range of 400 - 4000 cm^{-1}.

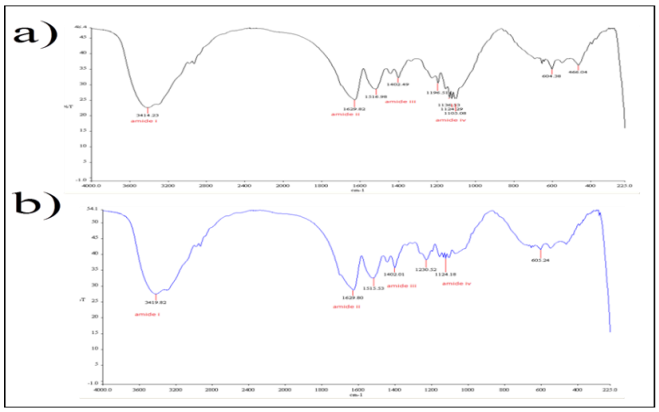

Figure 2. (a) FTIR spectrum of raw silk fibroin hydrogel. (b) FTIR spectrum of silk fibroin hydrogel loaded with fluconazole.

Scanning electron microscopy (SEM)

20 µL of silk fibroin hydrogel suspension in water was added directly on top of a conductive tape mounted on a SEM sample stub. The samples were dried overnight in air and then sputtered with platinum. The morphologies of silk hydrogels were imaged at a voltage of 15 kV at room temperature using a Zeiss Supra 55 VP SEM (Carl Zeiss SMT) in the Department of Glass and Ceramics, Rajshahi University of Engineering and Technology.

Thermo-gravimetric analysis (TGA)

The thermal stability of hydrogel was characterized using a DTG-6H (Shimadzu). The amount of sample for each measurement was about 10 mg and all of the measurements were carried out under a nitrogen atmosphere and heated 30°C to 600°C at a heating rate of 10°C min^{-1} in the Central Science Lab, Rajshahi University.

Differential scanning calorimetry (DSC)

The thermal behavior of hydrogel was determined by means of differential scanning calorimetry (DSC) using a Malvern differential scanning calorimeter. The samples used weighed between 10 and 15 mg and were measured between the ranges of 20 to 70°C at a scanning rate of 10°C/min under nitrogen atmosphere in the Department of Chemistry, Rajshahi University.

Antifungal studies

The antifungal activity test was carried out according to the reported method (Fatema *et. al.*, 2018). *Aspergillus Niger* and *Trichoderma* were used in this test. The fungus was cultivated on malt extract agar medium and incubated at 30°C for antifungal experiments.

Results and discussion

The (FTIR) Spectroscopy of silk fibroin hydrogel and drug loaded fluconazole

The FTIR spectrum of silk fibroin hydrogel was shown in the figure 2(a). Due to the presence of the amide groups in silk fibroin protein structure, the characteristic absorbance peak seen around 1629.82cm^{-1} resulting of absorption peaks of the peptide backbone of amide I (C=O) group. The absorbance peak exhibited at 1516.98cm^{-1} is due to the presence of amide II (N─H) group existing in the silk fibroin structure (Chen *et al.*, 2012). These characteristic absorbance peaks indicated the existence of hydrogen bonded NH group in the silk fibroin hydrogel. The conformation of *B.mori* silk fibroin is characterized by the β-sheet absorption peaks observed at 1629.82cm^{-1} due to the effect of glycerol treatment on the cross-linked silk fibroin hydrogel. Figure 2(a) also demonstrated absorbance peaks at 1196.51 cm^{-1} and 1402.49 cm^{-1} due to the existence of carbonyl group (C─O) present in silk fibroin hydrogel (Chen *et al.*, 2012).

The FTIR spectrum of silk fibroin hydrogel loaded with fluconazole was shown in the figure 2(b). The absorbance band in the frequency of 1629.80 cm^{-1} was represented the enriched β-sheet structure in silk II form. The absorbance band in the frequency of 1515.53 cm^{-1} was ascribed random coil in figure 2(b) also demonstrated absorbance peaks at 1124.18 cm^{-1} to 1402.49 cm^{-1} due to the existence of carbonyl group (C─O) present in silk fibroin hydrogel (Chen *et al.*, 2012). Moreover, the spectrum of fluconazole loaded SF hydrogel does not exhibit any characteristic differences relative to these of empty hydrogel. This might be the result of relatively very low ratio of fluconazole to silk fibroin protein (Ribeiro *et al.*, 2014).

The TGA analysis of silk fibroin hydrogel and drug loaded hydrogel

Silk fibroin hydrogel showed different thermo-gravimetric curves as in the figure 3(a). The initial weight loss below 100°C was due to the water

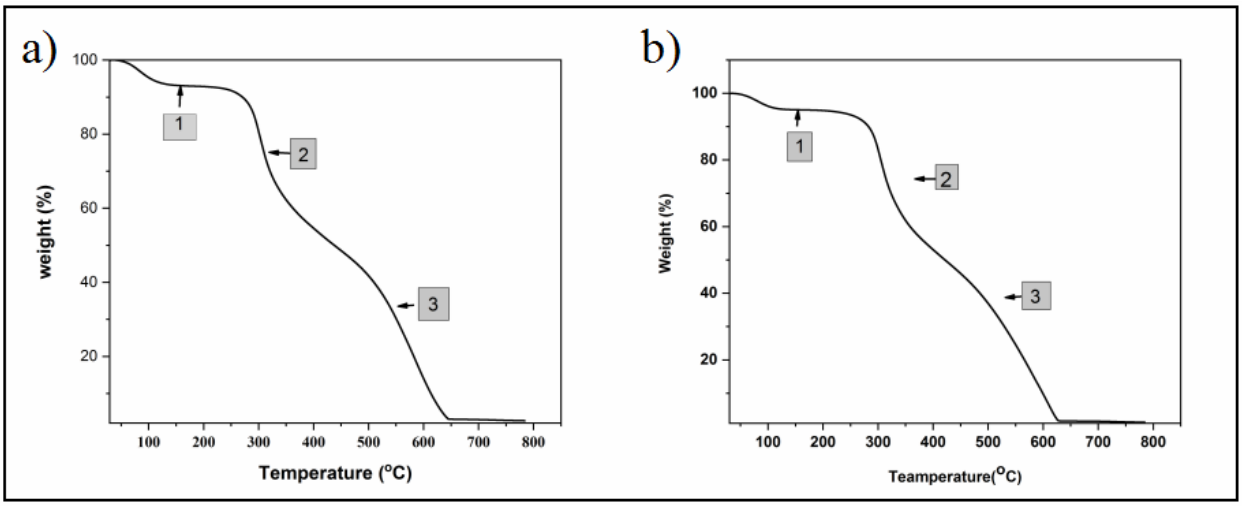

Figure 3. (a)TGA curve of silk fibroin hydrogel (b)TGA curve of drug(fluconazole) loaded hydrogel.

evaporation. At temperature above 200ºC, the weight loss occurred again. However, the silk did not completely decompose even at 700°C this is due the amorphous nature of silk fibroin. The result shows that silk fiber underwent of at least three thermal decomposition stages, which are 200 to 300°C, 300 to 350°C and 350 to 550°C. The decomposition at approximately 300°C is attributed to a disintegration of the intermolecular side chains during the crystalline melting process, while that at around 400°C is attributed to a main chain disintegration, coupled with simultaneous carbon atom rearrangements. It was reported that the decomposition at 300°C indicated the low crystallinity of the un-oriented β- type configuration. Therefore, it can be said that there is less possibility of obtaining a crystalline β-structure, which occurs in the temperature range of 325 to 330°C. It was found that the weight losses at 400°C were low for the gels due to low water content (Ribeiro et. al., 2014). A similar decomposition pattern is observed for silk fibroin hydrogel loaded drug 3(b). The rate of degradation is comparatively faster in this case. However, the curve of fluconazole loaded fibroin hydrogel does not exhibit any characteristic differences relative to these of empty hydrogel. This might be the result of relatively very low ratio of fluconazole to silk fibroin protein (Ribeiro *et al.*, 2014).

The DSC analysis of silk fibroin hydrogel

For the first heating, In the DSC curve at temperature 89.91°C showed that the β-sheet conformation of fibroin protein matrix was changing. This conformation was changed through the crystallization of fibroin from random coil to β-sheet (Ansary *et al.*, 2020).

The SEM analysis of silk fibroin hydrogel

Morphological features of silk fibroin hydrogels were studied at different magnifications using the scanning electron microscope (SEM). The effect of glycerol, propanol, ethanol was investigated in this study. For that, the gelation time was monitored by visual inspection of the samples. Hydrogels were formed after 1h, 2h, 5h for the formulations of F1, F2, F3 of glycerol, propanol and ethanol respectively. Among all the formulations, formulation F1 showed very faster gelation form. Glycerol can volume change of hydrogel by swelling while ethanol and propanol undergo transition between sol and gel phases (Hanawa *et al.*, 1995). Here glycerol might play a well swelling agent comparatively propanol and ethanol to diffuse drug into protein matrix. Glycerol might form strong intermolecular hydrogen bonds having three hydroxyl functional groups with fibroin protein matrix due to its high viscosity and low mobility. On the other hand, low viscous ethanol and propanol might form comparatively weaker hydrogen bond than glycerol (Guerette *et al.*, 1996) The higher extent of hydrogen bonding using glycerol makes stronger plasticizer than ethanol and propanol with increasing glycerol content considerably decreased gelation time of silk fibroin hydrogel was observed. It was observed more defined and smaller pores by increasing glycerol content in the hydrogels, which might be a result of faster gelation. Despite being smaller, large number of pores and an

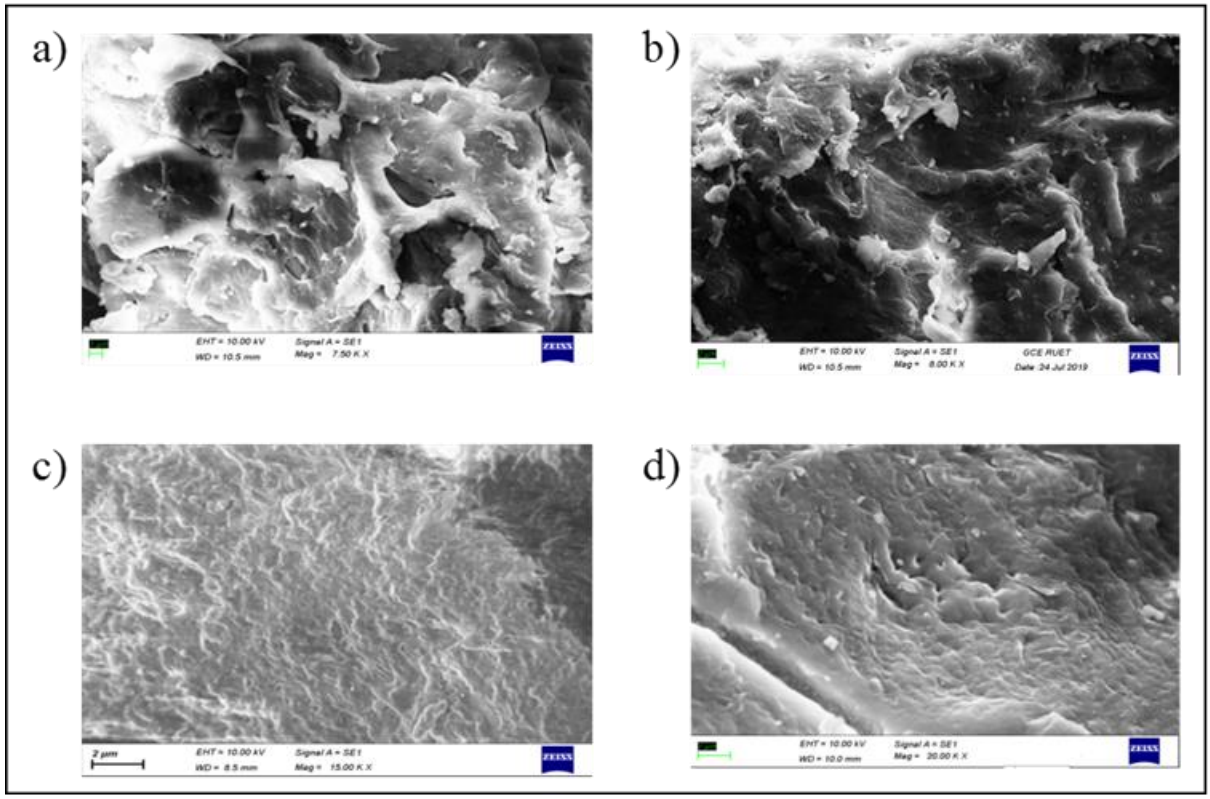

Figure 4. (a) Micrograph of fracture surface of fibroin hydrogel (b)Micrographs of porous surface of fibroin hydrogel. (c) Micrograph of the surface incorporated fibroin hydrogel with fluconazole (d) Micrograph of the porous surface incorporated fibroin hydrogel with fluconazole.

interconnected network were observed for hydrogel prepared using ethanol content (Matsumoto *et al.*, 2006). Morphologically, the silk fibroin hydrogel loaded with fluconazole showed a sponge-like cross-linked structure which was presented in figure 4(c). The hydrogel was produced by physical entanglement as well as chemical hydrogen and covalent bonding. Glycerol-stable hydrogels were formed from silk fibroin aqueous solutions after 3h of the formulation F4 which leads to the formation of porous matrices 4(b). This was due to induction of a sol-gel transition in the concentrated solution sample. The silk fibroin aqueous solution was converted into hydrogel. The process was induced by adding few drops of glycerol as crystallinity inducing solvent. The silk hydrogel intact in shape was formed and stabilized due to inter networking. The gel does not lose its integrity when kept vertically.

In vitro release of model drug from fibroin hydrogel

In order to investigate the applicability of silk fibroin hydrogel as a drug delivery system, hydrophobic drug fluconazole was loaded with silk fibroin hydrogel. The

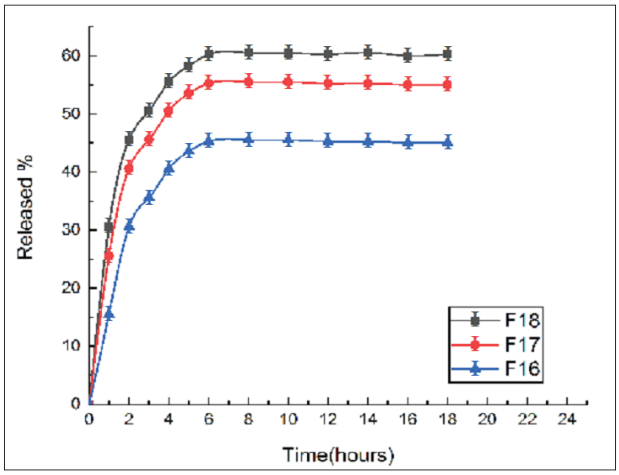

Figure 5. Released fluconazole (%) depending on soaking time.

in vitro release behavior of the model drug (fluconazole) was shown in the figure (5). The formulations of F16, F17 and F18 showed initial burst release of 50±3.52%, 45±3.26%, 35±2.45% for first three hours. After that, the sustained and cumulative

released within 20 hours was recorded as 60±1.65%, 55±2.12%, 45±1.78%, respectively. The drug release reached at equilibrium after six hours. By contrast, glycerol showed slow release indicating that a more sustained release has been achieved in the fibroin hydrogels. This is because glycerol might good plasticizer. Drug molecules might be adhered to the surface by diffusion into the protein matrix. The encapsulation efficiency was 85.5±1.67%, 73.4±1.76%, 71.6±1.35% for the formulation of F16, F17, F18 respectively.

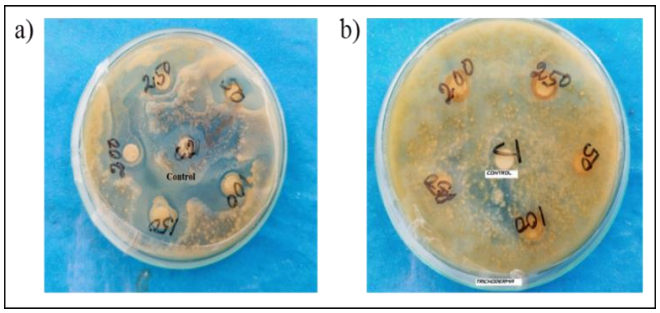

Figure 6. (a) Silk fibroin hydrogel loaded with fluconazole against pathogen of *Aspergillus niger* and its inhibition zone (b) Silk fibroin hydrogel loaded with fluconazole against pathogen of *trichoderma* and its negative inhibition zone.

Antifungal activity of the silk fibroin loaded with fluconazole

Antifungal susceptibility activity of silk fibroin hydrogel loaded with fluconazole was tested against control model drug fluconazole using a well diffusion assay. The diameter of inhibition zone reflects the magnitude of susceptibility of fungus.

Table 3. Result of silk fibroin loaded with fluconazole against *Aspergillus Niger* and its inhibition area in mm.

Sample no.	Control Drug (fluconazole)	Zone of inhibition measured in mm					
		Quantity of hydrogel with fluconazole	50µL	100µL	150µL	200µL	250µL
01	9mm	-	9.5mm	10.5mm	12.5mm	16mm	18mm

The strains susceptible to silk fibroin hydrogel loaded with fluconazole exhibited different parameter zone of inhibition against control (drug). According to zone of inhibition *Aspergillus niger* exhibited the highest sensitivity toward silk fibroin incorporated with fluconazole (Figure 6). It took 3 days for showing inhibition zone. The antifungal property of silk fibroin hydrogel loaded with fluconazole was analyzed by measuring the inhibition zone as shown in the (Table 3). The prepared silk fibroin hydrogel loaded with fluconazole showed an effective antifungal activity against pathogen of *Aspergillus niger*. The result suggests that silk fibroin hydrogel loaded with fluconazole underwent an interaction with fungus cell and displayed the strong action against *Aspergillus niger* (Fatema *et al.*, 2018). The antifungal activity of drug incorporated silk fibroin hydrogels increases with increasing the concentration of drug in hydrogels (Figure 6). A better efficacy was observed when the concentration was 100 µg/mL. The antifungal properties of silk fibroin hydrogel loaded with fluconazole against pathogen of *Trichoderma* showed no inhibition zone with fungus cell as shown in figure7(b).

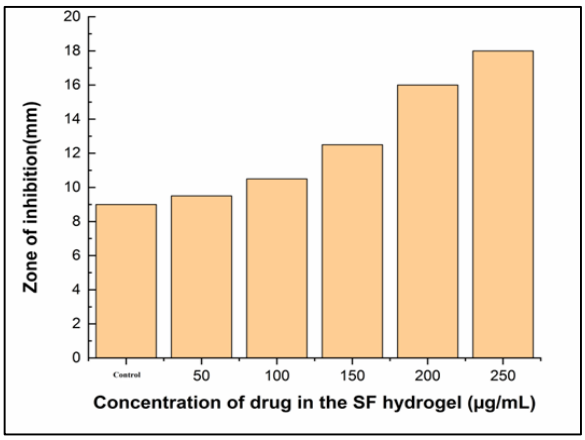

Figure 7. Statistical presentation of inhibition zone of silk fibroin hydrogel loaded fluconazole.

Conclusions

In this study, we have developed SF hydrogel from Bangladeshi silk and organic solvent to be applied as a carrier of fluconazole for controlled release applications. The SF hydrogels were successfully fabricated by a simple technique using ethanol, propanol, glycerol as a plasticizer. The initial burst release of drug in the hydrogel loaded with ethanol almost two time faster than hydrogel loaded with propanol. On the other hand, drug release in the hydrogel loaded with propanol almost two time faster than hydrogel loaded with glycerol. Though the drug release experiments were performed at the same conditions. After 6 hours all the hydrogel show equilibrium release.

Acknowledgements
Authors would like to thank Department of chemistry, Rajshahi University and Dean faculty of science for financial assistance during this research.

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