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Research Article

Silk Fibroin Hydrogel-Assisted Controlled Release of Antifungal Drug Ketoconazole

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Abstract

This research focused on preparing a ketoconazole-infused silk fibroin hydrogel to enhance patient compliance, leveraging the hydrogel's biocompatibility and biodegradability. The process used a 50% (v/v) ethanol, propanol, or glycerol solution treated with 2% (w/v) silk fibroin at 37°C. The use of glycerol quickened the gelation process. The hydrogel's structure was analyzed via SEM, and UV-VIS spectrometry was used to evaluate the encapsulation efficiency and ketoconazole release profile. The surface structure varied based on the formulation and the best encapsulation efficiency was observed with ethanol (60±1.78%), followed by propanol (56±2.72%) and glycerol (47±1.42%). The release profile demonstrated an initial burst release followed by 20 hours of sustained release. Furthermore, the hydrogel-encapsulated drug demonstrated effective antifungal activity against *Aspergillus Niger*. As a result, silk fibroin hydrogels are a promising option for controlled topical delivery of ketoconazole.

Keywords: Silk fibroin, Controlled release, Hydrogels, Drug delivery.

INTRODUCTION

Silk is a natural protein fiber derived from the Silkworm, Bombyx mori and some insects. Silk fibroin is interest for its potential use in areas like tissue engineering, enzyme immobilization, and drug delivery due to its unique properties like slow degradation and biocompatibility[1]. It can be processed into various forms such as nano-particles, microspheres, powders, hydrogels, and films. Fibroin's polymer structure is crucial for drug storage in a delivery device. Gelation of SF solutions is determined by the content of β-sheet, which impacts the drug release behavior from hydrogels[2]. Silk fibroin hydrogels are biocompatible and non-toxic, making them safe for use in medical applications[3]. Silk fibroin hydrogels are biodegradable, meaning they can be degraded into naturally occurring substances by the body over time [4]. Implantable polymer drug depots that provide controlled and sustained drug delivery offer a host of benefits compared to periodic systemic administration, such as improved efficacy and cost-effectiveness, decreased occurrence of unwanted side effects, heightened patient convenience and adherence, and consistent maintenance of drug levels within a therapeutic range without any fluctuations[5]. Protein-based nanoparticles are becoming an increasingly popular option for drug delivery systems due to their unique capabilities. These carriers are biodegradable, non-antigenic, and have exceptional biocompatibility[6]. Silk fibroin hydrogels are three-dimensional polymers formed from the sol-gel transition of silk fibroin solution in an aqueous solution with added organic solvent[7]. The gelation process is enhanced by factors such as increased concentration of protein, temperature, and organic solvent. The rate of gelation depends on the pH of the solution[8].

Hydrogel drug delivery systems use hydrogel for targeted delivery and controlled release of drugs, with applications in pharmaceutical and medical technology[9]. Silk fibroin from Bombyx mori is obtained by dissolving the cocoons in aqueous solutions of alkali and then spinning the resulting solution to form fibers[10]. The silk fibroin fibers can then be processed to produce various materials, such as fibers, films, and hydrogels[11].

Silk fibroin (SF) is a protein-based bio macromolecule with excellent biocompatibility, biodegradability and low immunogenicity[12]. The progress of SF-based nanoparticles for drug delivery have received significant attention due to high binding capacity for various drugs, controlled drug release properties and mild preparation conditions[13,14]. By adjusting the particle size, the chemical structure and properties, the qualified or recombinant SF-based nanoparticles can be programmed to improve the therapeutic efficiency of drugs

encapsulated into these nanoparticles[15]. Therefore, they can be used to deliver small molecule drugs (e.g. anti-cancer drugs), protein and growth factor drugs, gene drugs etc[16,17]. Silk fibroin hydrogels were incorporated with the antifungal drug ketoconazole for controlled drug release. The results showed that the hydrogels were able to sustain the release of the drug over a prolonged period of time and effectively treat fungal infections.

This study reports that silk fibroin hydrogels loaded with ketoconazole can achieve a controlled release profile, improving patient compliance.

MATERIALS AND METHOD

Materials

Matured Bombyx mori silk cocoons were collected from the Bangladesh Sericulture and Training Institute, Rajshahi, Bangladesh. Lithium Bromide (Fisher Scientific Company, USA), Petroleum Ether, Sodium carbonate, Phosphate Buffer Solution (PBS), Model drug (Ketoconazole) were used in this study.

Silk purification and preparation of Silk fibroin (SF) solution

To obtain sericin-free silk fibroin, 7.00g of cleaned white cocoon shells were degummed twice in a boiling solution of 0.02M Na_2CO_3 for one hour. The fibers were then spun at 175 rpm for three times in 2-hour sessions on a hot plate set at 70°C. Afterwards, the fibers were washed three times with boiling water and dried in an oven at 65°C for 50 minutes, yielding a 5.626g sample. The silk fibroin was stored in a desiccator for 2 days. A SF solution was made by dissolving 2.00g of dry silk fibers in 10 mL of 9.28 M LiBr solution. The solution was stirred continuously with a magnetic stirrer set at 80°C and 220 rpm until a clear solution was achieved. The fibroin solution was then transferred to a cellophane dialyzing bag and immersed in 200 mL of distilled water for at least 3 days. After dialysis, the solution was centrifuged at 20,000 rpm for 15 minutes and freeze-dried overnight. The dried sample was used for hydrogel preparation[18].

Preparation of Silk Fibroin Hydrogels

Silk fibroin hydrogels were prepared using 4% (w/v) silk fibroin solution by adding 50% (v/v) glycerol, propanol or ethanol at 37°C. The composition of each hydrogel is shown in (**Table 2.1**). The mixed solutions (20 mL) were poured into molds (4 cm diameter) and left on a thermostatic bath at 37°C until gelation occurred. Afterward, the gels were gently washed with distilled water[4].

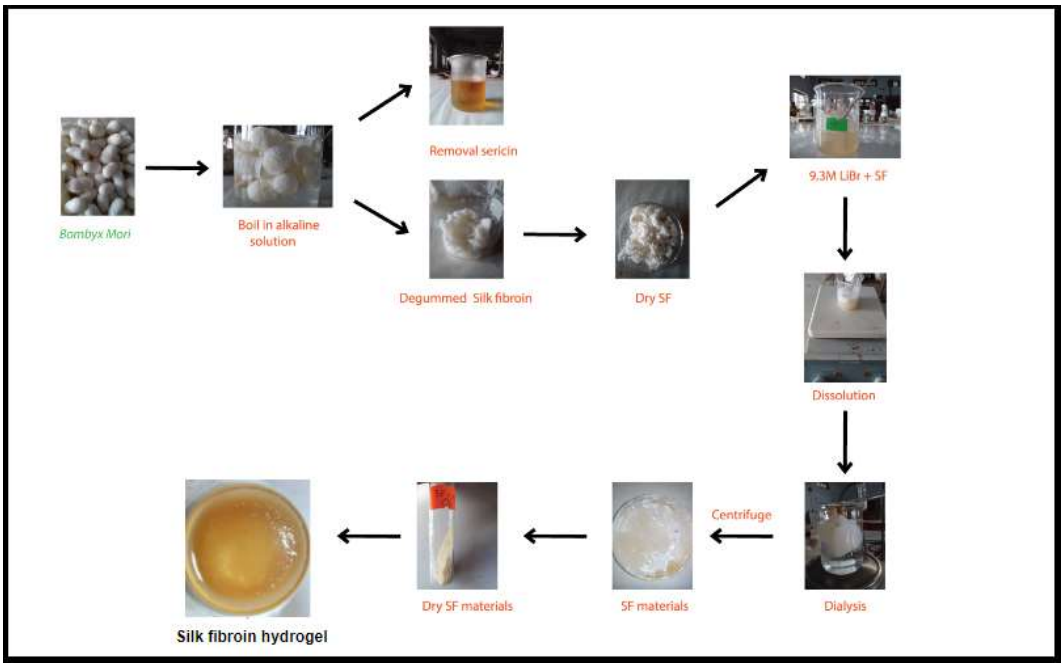

Figure 1: Fabrication of Silk fibroin hydrogel

Figure 2: Silk fibroin hydrogels prepared in formulations: (a) F7 and drug loaded hydrogel in formulation of (b) F19

Table 2.1: Composition and gelation time of silk fibroin hydrogels prepared in different formulation.

Formulation No.	Volume of silk fibroin solution (mL)	Volume of glycerol solution (mL)	Volume of propanol solution (mL)	Volume of ethanol solution (mL)	Volume 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 (Ketoconazole) into fibroin hydrogel

To encapsulate ketoconazole in silk fibroin hydrogel, the fibroin solvent ratio of 1:3 was found to maintain the best integrity. To evaluate the loading efficiency of ketoconazole, the volume ratio of 1:3 was also tested. To incorporate ketoconazole, (0.2g/mL) of a 10 mL dialyzed fibroin solution was added to a 0.01g/mL solution of ketoconazole. The mixture was then combined with 10mL of either 50% glycerol, propanol, or ethanol and placed in 4 cm diameter molds on a thermostatic bath at 37^{o}C until gelation occurred. The resulting gel was washed gently with distilled water twice[4,18].

Table 2.2: Composition of silk fibroin loaded with Ketoconazole.

Formulation No.	Volume of silk fibroin solution (mL)	Volume of Ketoconazole solution (mL)	Volume of glycerol solution (mL)	Volume of propanol solution (mL)	Volume 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

Characterizations

Encapsulation efficiency of Ketoconazole in Silk fibroin hydrogel

Encapsulation efficiency was calculated using UV-Vis spectroscopy. A calibration curve for the model drug (with a wavelength of 257 nm) was generated using five different concentrations of stock solutions. 10 mL of 0.2g/mL dialyzed silk fibroin solution was mixed with 1 mL of 0.02g/mL Ketoconazole solution before gel formation. Then, 5 mL of either 50% (v/v) glycerol, propanol, or ethanol was added to the mixture, respectively, before gelation. The supernatant was then removed and analyzed for residual ketoconazole concentration using UV-Vis spectroscopy (wave length of 257 nm). The standard calibration curve of ketoconazole solution was used to quantify the drug[19]. The amount of supernatant was used to calculate the amount of drug incorporated into the silk fibroin hydrogel. The experiments were performed twice and encapsulation efficiency was determined by the equation [20].

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

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

In vitro released studies of drug from Silk Fibroin hydrogels

Silk fibroin hydrogels containing ketoconazole were immersed in 50 mL of phosphate buffer solution (PBS) at p^H 7.4 followed by incubation at 37^{0}C 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 257nm)[21].

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[22].

Antifungal studies

The antifungal activity test was carried out according to the reported method. *Aspergillus Niger* was used in this test. The fungus was cultivated on malt extract agar medium and incubated at 30^{o}C for antifungal experiments in the Department of Botany, Rajshahi University[23].

RESULTS AND DISCUSSION

Morphological Studies of Silk Fibroin Hydrogels

In this study, different dehydrating solvents (ethanol, propanol, and glycerol) were added to silk fibroin dialyzed solutions to prepare silk fibroin hydrogels. The addition of the solvents altered the silk fibroin's molecular interactions and caused a conformational change, leading to gelation. The effect of each solvent on gelation time was analyzed through visual inspection[24].The results showed that hydrogels formed with a 1:3 fibroin-solvent ratio (F1, F2, and F3) were fragile, while those with a 3:1 ratio (F7-F9) were rigid and easily collapsed. Hydrogels formed with glycerol as the cross-linker showed a considerably reduced gelation time due to the increased hydrophobicity of the protein[14].The solvent had a significant impact on the structure and surface morphology of the hydrogels, with glycerol leading to a more porous and homogeneous structure compared to ethanol and propanol. So, the mechanical properties of the hydrogels were influenced by the solvent, with the hydrogels in glycerol showing improved mechanical stability compared to those in other solvent[10,25]. The surface morphology of the hydrogels was observed through SEM and showed a smooth, cross-linked structure as a result of the accelerated gelation process [26,27].

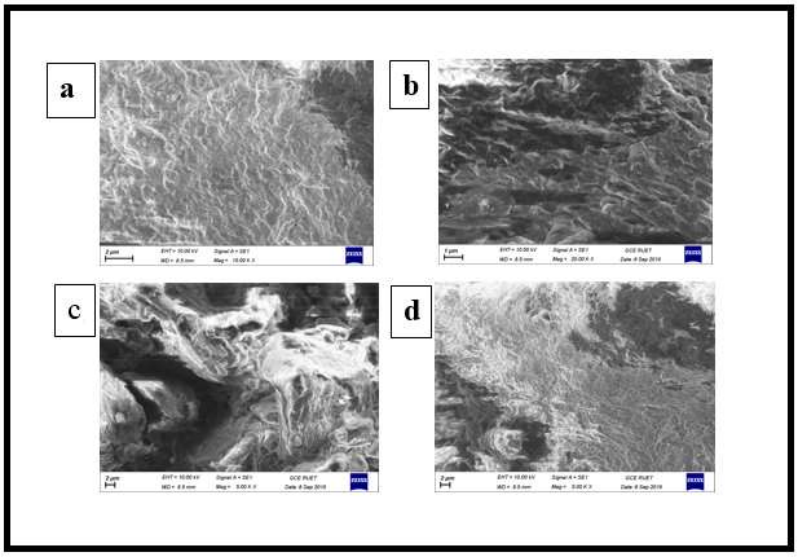

Figure 3: SEM images of silk fibroin hydrogels prepared in formulations (a) F15, (b) F14, (c) F13 (d) F19

In Vitro Release Profiles of Ketoconazole from Silk Fibroin Hydrogels

The release of ketoconazole from silk fibroin hydrogels in PBS (pH 7.4) was studied and depicted in (**Figure 4**). The initial burst release was 32±3.25% for F21, 30±3.56% for F20, and 29±2.32% for F19 in the first three hours. Afterwards, a sustained release was observed and the cumulative release reached 60±1.78% for F21, 56±2.72% for F20, and 47±1.42% for F19 over the next 20 hours. Ethanol is a polar solvent that can cause silk fibroin to denature and form β-sheet structures. It can also lead to the formation of pores in the silk fibroin matrix, which can be useful for applications that require high porosity, such as tissue engineering scaffolds. Propanol is also a polar solvent that can induce silk fibroin to form β-sheet structures. However, it is less effective than ethanol in promoting the formation of pores in the silk fibroin matrix. Glycerol can plasticize silk fibroin, making it more flexible and less brittle. Glycerol can also increase the water content of the silk fibroin matrix, which can improve its biocompatibility and cell adhesion properties[7,28].The hydrogel with glycerol showed a low initial burst release and prolonged sustained release due to its viscous nature[4,21]. The low mobility of ketoconazole to the release medium might be attributed to the highly viscous glycerol[29]. Furthermore, ketoconazole dissolved in glycerol showed reduced gelation time, confirming glycerol's effect in accelerating fibroin gelation. This can be explained by glycerol transforming the well-organized β-sheet structures of fibroin into a cross-linked process, resulting in a more controlled drug release[30].

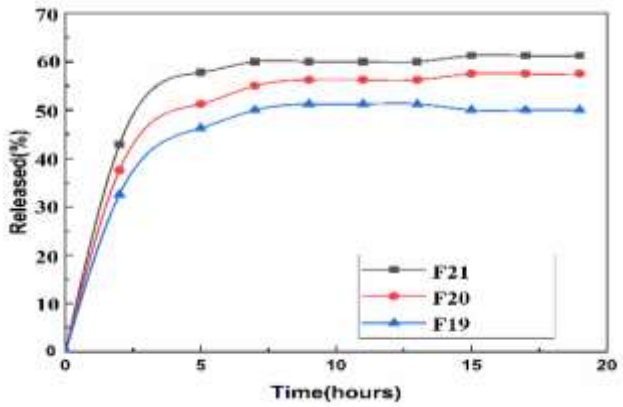

Figure 4: Released Ketoconazole (%) depending on soaking time.

Antifungal Activity of Ketoconazole Loaded Silk Fibroin Hydrogels

The antifungal activity of ketoconazole loaded silk fibroin hydrogels was evaluated using the well diffusion method[31].The effectiveness of these hydrogels against fungal strains was determined by measuring the size of the inhibition zones, which indicates the susceptibility of the fungus. The results showed that *Aspergillus Niger* was highly susceptible to ketoconazole-incorporated silk fibroin hydrogels and displayed a noticeable inhibition zone after 3 days[32].This suggests that the ketoconazole loaded hydrogels effectively interacted with fungal cells and strongly inhibited the growth of *Aspergillus Niger*. The antifungal activity of these hydrogels improved with increasing ketoconazole concentration, with a maximum efficacy observed at a concentration of 250 µg/mL. However, there was no significant improvement in efficacy beyond this concentration.

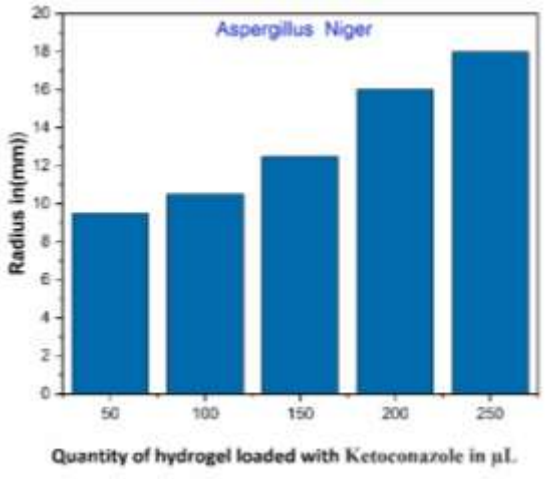

Figure 5: Statistical presentation of inhibition zone of silk fibroin hydrogel loaded Ketoconazole.

CONCLUSION

In this study, an eco-friendly method was used to prepare ketoconazole loaded silk fibroin hydrogels. The prepared ketoconazole were incorporated into silk fibroin hydrogels, and their properties were evaluated, including morphology, encapsulation efficiency, and in vitro release profile. The results showed that glycerol-containing formulation F19 had better efficiency and controlled release of ketoconazole. Additionally, the antifungal activity results showed a promising response against *Aspergillus Niger*. Overall By incorporating ketoconazole into the hydrogel matrix, the drug release can be controlled and sustained over a specific period of time. This approach has potential applications in the treatment of fungal infections and can help improve the efficacy and reduce the toxicity of ketoconazole.

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Ethics declarations

Since we only conducted an in vitro studies, no ethical declaration is required for this study.

Author Contributions

Tomal Chandro Roy-experimental design and data analysis and manuscript writing.

Protyasha Biswas, Md Ali Haider also assisted antifungal activity test.

Rezaul Haque Ansary-manuscript correction and proof reading.

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Conflicts of interest

With regard to the research and publication of this article, the author declares that there are no potential conflicts of interest.

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