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Investigation of C−C Bond Formation Mediated by *Bombyx mori* Silk Fibroin Materials

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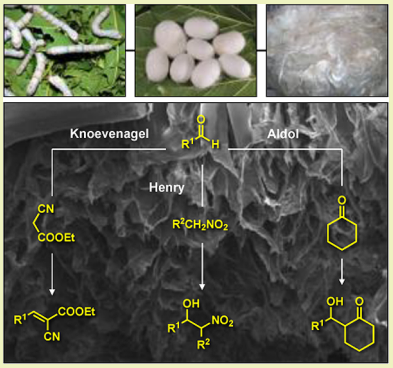
Supporting Information

ABSTRACT: The formation of C−C bonds is a prerequisite for all life on earth. Understanding the role of proteins in mediating the formation of these bonds is important for understanding biological mechanisms in evolution, as well as for designing "green catalysts". In this work, the ability of silk fibroin (SF) proteins to mediate selective C−C bond formation under mild conditions was comprehensively evaluated and compared between different SF-based materials and other proteins. Aqueous SF solution (ASFS), freeze-dried SF (FDSF), mesoporous SF (MPSF), and SF hydrogel (SFHG) materials were prepared and characterized by a variety of techniques including, among others, FE-SEM, ICP-OES, FT-IR, and TGA. The nitroaldol (Henry) reaction, Knoevenagel condensation, and direct aldol reaction were used as models for this study, in which the recovery and reusability of the protein was also evaluated.



KEYWORDS: *Silk fibroin, C−C Bond formation, Proteins, Aldol-like reactions, Knoevenagel condensation*

INTRODUCTION

The formation of C−C bonds is of central importance in modern chemistry and a prerequisite for all life on earth. Understanding the role of natural proteins in promoting the formation or cleavage of C−C bonds may help to find missing links in human evolution and the mechanisms of action of biological systems. On the other hand, the growing environmental concerns coupled with stricter regulations are driving a gradual shift from the use of petrochemical-based feedstocks to environmentally friendly resources and processes.[1] In this sense, the successful development of protein-based catalysts that are biocompatible and robust and can be mass-produced, which can be very important for designing "green catalysts".

Motivated by the above combined visions, we have recently found that the morphology and/or physical state of several biopolymers and proteins plays a significant role in the kinetics of some C−C bond forming reactions.[2,3] Within this context, silk fibroin (SF) constitutes another protein of great interest because it is a water-soluble protein with hydrophobic domains that could solubilize organic substrates. Moreover, we found especially advantageous the possibility of preparing SF materials in different physical forms (e.g., films,[4] porous scaffolds,[5] gels,[6,7] mats[9]), so the effect of these forms on the chemical reactivity could be studied. SF has a high molecular weight (about 390 kDa), and it is obtained from the cocoons of the larvae of the mulberry silkworm (*Bombyx mori*). In general, SF is composed of three major proteins, a heavy-chain fibroin (H-chain, about 325 kDa), a light-chain fibroin (L-chain, about 25 kDa), and an accessory glycoprotein (P25 protein, about 30 kDa). Glycine (Gly), alanine (Ala), serine (Ser), and tyrosine (Tyr) are the most common amino acids in SF resulting in a total content of about 90 mol %. Repeating units of Gly−Ala−Gly−Ala−Gly−Ser/Tyr forms the core of the protein, and the strong hydrophobicity of these amino acids results in van der Waals interactions that drives SF to self-assemble into highly crystalline antiparallel β-sheet structures. The silk polymorphs include silk I, silk II, and an air/water interfacial silk III.[9−14]

A unique combination of nontoxicity, biocompatibility, tunable biodegradation rates, availability, malleability, and excellent mechanical strength has positioned SF proteins as versatile materials for a number of applications. Besides the traditional use in textile industry and in biomedicine (e.g., clinical sutures,[15] drug delivery vehicles,[16−18] bone,[19−21] cartilage,[22,23] fat,[24] ligament,[25] and vasculature[26−28] engineering scaffolds), SF has been also successfully employed as support for a variety of metal-based catalysts. In this regard, metal nanoparticles grafted onto the surface of SF fibers have been used as heterogeneous catalysts for several reactions including hydrogenations,[29−31] hydroxylation of phenol,[32] reduction of *p*-nitrophenol,[33] and oxidation of methanethiol and hydrogen sulfide.[34]

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Herein, we report a comparative study to identify the efficacy of SF in promoting C−C bond forming reactions (i.e., aldol reaction, nitroaldol (Henry) reaction, Knoevenagel condensation). Because SF can be assembled into various physical forms such as mesoporous silk or hydrogels, we also demonstrate the effect of the physical nature of SF on its ability to catalyze these reactions.

EXPERIMENTAL SECTION

Materials and Methods. ^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 300 spectrometer. Chemical shifts are denoted in δ (ppm) relative to tetramethylsilane (TMS δ = 0) as an internal standard or relative to residual solvent peaks. Reaction products were analyzed by chiral-phase HPLC using a Varian 920-LC liquid chromatograph. The columns Phenomenex Lux Cellulose-1, 4.6 mm × 250 mm, 5 μm, and AS-H, 4.6 mm × 250 mm, 10 μm, were used for the analysis of the nitroaldol and aldol products, respectively. TLC was facilitated by the use of the following stains in addition to UV light (254 nm) with fluorescent-indicating plates (aluminum sheet precoated with silica gel 60 F254, Merck): phosphomolybdic acid, vanillin, and iodine. FT-IR spectra were recorded using a Bio-Rad FT-IR Excalibur FTS 3000 equipped with a Diamond ATR (attenuated total reflection) accessory (Golden Gate). The morphology of the materials was observed with a Carl Zeiss Merlin field-emission scanning electron microscope (FESEM, resolution 0.8 nm) equipped with a digital camera and operating at 5 kV (accelerating voltage) and 10 μA (emission current). Xerogel samples of the corresponding hydrogels were prepared by the freeze-drying (FD) method. The resulting material was placed on top of a tin plate and shielded by Pt (40 mA during 30 s; film thickness ≈5 nm). Thermal gravimetric analysis (TGA) measurements were carried out under nitrogen with the following program heating rate: (1) equilibration step for 30 min @ 25 °C, (2) heating profile from 25 to 800 °C @ 10 °C/min, and (3) 15 min @ 800 °C. The lithium content of the samples was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Spectro Analytical Instruments ICP Modula EOP. Analytical-grade solvents and commercially available reagents were purchased from Merck, TCI Europe, or Sigma-Aldrich and were used as received without further purification. Pure H_2O (Milli-Q) was used in all water-included experiments. Cocoons of the *Bombyx mori* (silkworm of the mulberry tree) for this work were provided by the Central Sericulture Training and Research Institute (Mysore, India) and were degummed at the Physical/Materials Chemistry Division, National Chemical Laboratory, Pune, India.

Preparation of Silk Fibroin Materials. The materials for reactivity tests were prepared according to the general procedures reported in the literature with slight modifications.

Preparation of Regenerated Aqueous Silk Fibroin Solution (ASFS). Silk cocoons from *Bombyx mori* were boiled for 1 h in a 0.5 wt % aqueous solution of $NaHCO_3$ to remove sericin. The remaining fibroin fibers were washed thoroughly with excess of H_2O to remove $NaHCO_3$. The dried silk fibroin fibers were dissolved during 24 h at room temperature in an aqueous LiBr solution (41 g of LiBr in 30 mL Milli-Q H_2O) stored in a plastic screw cap bottle. The resulting solution was further dialyzed against Milli-Q H_2O for 48 h at 4 °C using a dialysis tube of cellulose acetate membrane with a molecular weight cutoff (MWCO) of 12 kDa. The H_2O was exchanged five times, first after 3 h, then 5 h, and later after 12 h each. After the dialyzed solution was centrifuged at 4000 rpm for 40 min at room temperature, the supernatant was collected. This silk fibroin solution had a final concentration between 2.9 and 5.4 wt %, which could be stored in a plastic screw cap bottle at 4 °C for about 1 month. Concentration of the regenerated silk fibroin solution was determined by gravimetric analysis and by measuring the absorption at λ_{max} = 272 nm (ε = 11.8 mol L^{-1} cm^{-1}).

Preparation of Freeze-Dried Silk Fibroin (FDSF). The pH of the obtained ASFS was adjusted to 7. The precipitates formed during neutralization were removed by centrifugation at 4000 rpm for 20 min. The supernatant collected and freeze-dried for 3 days to obtain FDSF. For reactivity studies, bulk FDSF was cut into 10 mg pieces.

Preparation of Mesoporous Silk Fibroin (MPSF). Thirty milliliters of freshly prepared ASFS was transferred to a slide-a-lyzer dialysis cassette with a MWCO of 3500 (capacity 12−30 mL) and then concentrated by dialysis against an aqueous 25 wt % PEG 20000 solution for about 8 h in total at room temperature. Additional 10 mL of ASFS was added after 3 h to the dialysis cassette. The concentrated ASFS (CASFS) had a final concentration of 30−40 wt %. CASFS was diluted to a 17 wt % silk solution, and the pH was adjusted to neutral pH (pH test paper) prior to combination with 5 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The obtained viscous solution (1 g) was transferred to a Teflon mold, in which NaCl (10 g) was furnished. The mold was covered with a perforated aluminum foil. The setting was left standing for 24 h at room temperature. After this time, the solid film was matured in MeOH for 30 min to obtain the β-sheet structure (MPSF). Finally, the film was washed with Milli-Q H_2O and dried under air. For reactivity studies, bulk MPSF was ground into a fine powder.

Preparation of Silk Fibroin Hydrogel (SFHG).[35,36] The pH of freshly prepared ASFS was adjusted to 5. The precipitates formed during neutralization were removed by centrifugation at 4000 rpm for 20 min. The supernatant was diluted to 20 g L^{-1}, and 0.5 mL of this solution (10 mg silk fibroin) was transferred to a screw cap vial (4 mL) and left standing for 2 days at room temperature to obtain stable and white hydrogel (SFHG). This gel was found to remain stable for several days in the presence of additional water or organic solvents like MeOH, DMSO, or THF.

Reactivity Studies. *Typical Procedure for the FDSF-Mediated Nitroaldol (Henry) Reaction.* To a mixture of 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and nitromethane (54 μL, 1 mmol) in DMSO (0.5 mL) placed in a screw cap vial (4 mL), FDSF (10 mg) was added in one portion. The resulting reaction mixture was gently stirred for 4 h at room temperature. After this time, 1 mL of EtOAc was added, and the diluted supernatant solution was removed by decantation. The remaining catalyst was washed with EtOAc (3 mL × 1 mL, for 5 min in each cycle). The combined organic phases were washed with H_2O (2 mL × 5 mL) and brine (5 mL), dried over anhydrous Na_2SO_4, filtrated, and evaporated under reduced pressure to obtain the crude product. To determine the NMR yield, the crude product was dissolved in 1 mL of $CDCl_3$, and diphenylmethane (16.7 μL, 0.1 mmol) or *N*,*N*-dimethylacetamide (9.2 μL, 0.1 mmol) were added as the internal standard. Note that the same procedure and same amount of catalyst (10 mg) was used for testing other silk-based materials. In the case of ASFS, the product was extracted directly from the aqueous phase.

Typical Procedure for the FDSF-Mediated Knoevenagel Condensation Reaction. To a mixture of 2-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and ethyl cyanoacetate (11.7 μL, 0.11 mmol) in DMSO (0.5 mL) placed in a screw cap vial (4 mL), FDSF (10 mg) was added in one portion. The resulting reaction mixture was gently stirred for the given time at room temperature. The work-up of the reaction, washing of the catalyst, and determination of the NMR yield were carried out as described for the FDSF-mediated nitroaldol reaction.

Typical Procedure for the FDSF-Mediated Direct Aldol Reaction. To a mixture of 4-nitrobenzaldehyde (15.1 mg, 0.1 mmol) and cyclohexanone (208 μL, 2.0 mmol) in DMSO (0.5 mL) placed in a screw cap vial (4 mL), FDSF (10 mg) was added in one portion. The resulting reaction mixture was gently stirred for the given time at room temperature. The work-up of the reaction, washing of the catalyst, and determination of the NMR yield were carried out as described for the FDSF-mediated nitroaldol reaction.

Typical Recycling Experiment for the FDSF-Mediated Nitroaldol (Henry) and Knoevenagel Condensation Reactions. Method A: After quenching the reaction with EtOAc, the catalyst was washed as previously described, and the remaining catalyst was dried under high vacuum to remove residual solvent. The dried catalyst was then added to the appropriate mixture of substrates in DMSO. This procedure was repeated for all further cycles. The work-up and determination of the yield was accomplished as described for the FDSF-mediated nitroaldol

reaction. Method B: The reaction was quenched by addition of 1 mL of DMSO (instead of EtOAc). The liquid of the reaction mixture was decanted, and the remaining catalyst was washed with DMSO (3 mL × 1 mL) and used in the next cycle of the reaction. The combined organic layers were diluted with EtOAc (36 mL) and washed with H_2O (2 mL × 36 mL) and brine (36 mL), dried over anhydrous Na_2SO_4, filtrated, and evaporated under reduced pressure to obtain the crude product. The NMR yield was determined as described in the representative procedure for the FDSF-mediated nitroaldol reaction.

RESULTS AND DISCUSSION

Preparation of SF-Based Materials. The general procedures reported in the literature were used to prepare a variety of silk-based materials (see Experimental Section). Thereby, cocoons of the silkworm *Bombyx mori* were first boiled in aqueous $NaHCO_3$ solution (0.5 wt %) for 1 h to remove sericin. Silk fibroin fibers (SFF) were isolated after a washing protocol to remove salt residues and subsequent drying under air (Figure 1A). This material was further

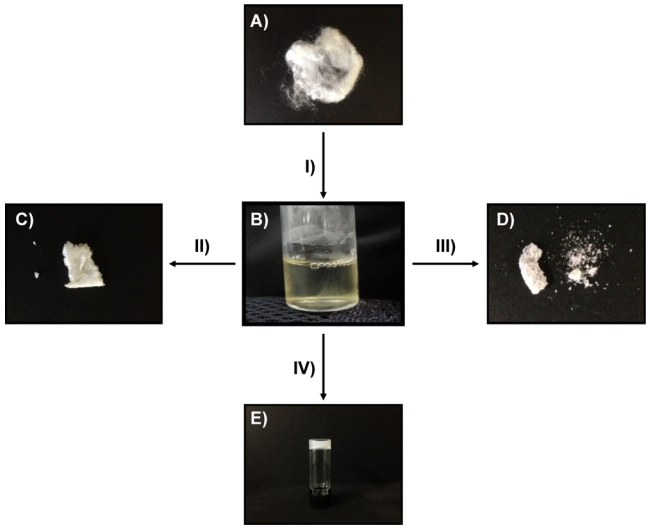


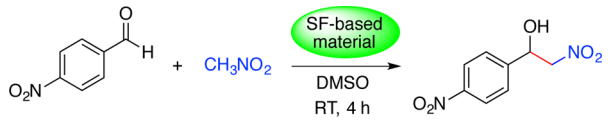
Figure 1. Overview of silk fibroin-based materials used in this work: (A) SFF, (B) SFAS, (C) FDSF, (D) MPSF, and (E) SFHG. Conditions: (I) aq. LiBr solution (15.7 M), 24 h, room temperature. (II) Freeze-drying of 2.9−5.4 wt % SFAS, pH 7, 3 days. (III) (i) 17 wt % SFAS, HFIP and (ii) NaCl, MeOH. IV) 0.5 mL of a 20 g L^{-1} SFAS, pH 5, 2 days, room temperature.

dissolved under basic conditions and extensively dialyzed against water to obtain a highly pure aqueous silk fibroin solution (ASFS) (Figure 1B) in a range between 2.9 and 5.4 wt %. Neutralization of the slightly basic ASFS with diluted HCl and subsequent freeze-drying afforded the FDSF material as a white solid after 3 days (Figure 1C). Mesoporous silk fibroin (MPSF) was prepared by dissolving FDSF in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain a viscous 17 wt % silk solution, which was then combined with NaCl that acted as porogen. HFIP was allowed to evaporate overnight, and the obtained residue was matured in MeOH to induce the formation of the insoluble β-sheet structure. This obtained material was extensively washed with water to remove possible salt impurities affording the corresponding MPSF material as a white solid (Figure 1 D). Finally, a silk fibroin opaque hydrogel (SFHG) was formed at room temperature from a silk solution (c = 20 g L^{-1}) at pH 5 (Figure 1E). The slightly acidic pH was chosen to form stable and neutral hydrogel material at the given concentration. Characterization of the above materials by FT-IR and TGA was fully consistent with previous reports in the literature (see Experimental Section).

Nitroaldol (Henry) Reaction. Nitroaldol (Henry) reaction is a classical and valuable synthetic method for the synthesis of β-nitroalcohols by combination of nitroalkanes with carbonyl compounds (i.e., aldehydes, ketones) in the presence of an ionic or nonionic base-catalyst.[37,38] However, one of the main drawbacks of this reaction is the formation of several side products that usually complicate the isolation of the desired compounds. These side products include mainly polymerizable nitroalkenes formed upon dehydration of the β-nitroalcohols, self-condensed products especially with sterically hindered substrates (i.e., Cannizaro reaction), epimerized β-nitroalcohols, and products derived from the Nef reaction.[37] In general, with aromatic aldehydes, the selectivity of the reaction is strongly influenced by the electronic nature of the substituents and their ability to favor either the imine or ion-pair mechanism.[37]

In our previous studies with other biopolymers and proteins,[2,3] we demonstrated that the morphology and/or physical state of a protein catalyst plays a significant role in the kinetics of aldol-like reactions under heterogeneous or semi-heterogeneous conditions. Motivated by this finding, we carried out a comparative evaluation of the effect of the SF-based materials (i.e., ASFS, FDSF, MPSF, and SFHG) in the nitroaldol (Henry) model reaction between 4-nitrobenzaldehyde (**1**) and excess of nitromethane (**2**) in DMSO at room temperature over a period of 4 h (Table 1). Preliminary experiments established an optimal use of 10-fold molar excess of nitromethane equivalents for this transformation and 10 mg of catalyst per 0.1 mmol of aldehyde (higher amounts of silk did not improved the yields). The results showed that FDSF catalyzed the formation of the corresponding β-nitroalcohol **3a**

Table 1. Screening of Different Silk-Based Materials in the Nitroaldol (Henry) Reaction[a]



1a 2 3a

entry	SF-based material	yield **3a** (%)[b]
1	FDSF	95
2	FDSF	0 (control)[c]
3	MPSF	4
4	MPSF	88[d]
5	MPSF	0 (control)[e]
6	SFHG	0[f]
7	SFHG	83[g]
8	ASFS	22[h]

[a]Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 0.1 mmol), nitromethane (**2**, 1.0 mmol), SF material (10 mg), DMSO (0.5 mL), 4 h, room temperature. [b]Determined by ^{1}H NMR analysis of the crude product based on aldehyde proton. Reported yields correspond to the average values of two independent experiments (STDV = ± 2). [c]Control experiment performed in the absence of SF material; reaction time = 4 h. [d]Reaction time = 48 h. [e]Control experiment performed in the absence of SF material; reaction time = 48 h. [f]Reaction mixture was layered on top of the hydrogel. [g]Reaction mixture was combined with gel pieces and stirred. [h]Silk protein precipitated during the reaction.

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with an excellent yield of 95% (entry 1). Comparable results were obtained when the reaction was scaled up 10 times. In sharp contrast, MPSF displayed much slower kinetics as reflected by the very low yield of **3a** (4%) within the same period of time. This yield could be enhanced to 88% upon extension of the reaction time to 48 h (entry 4). The bulk SFHG provided negligible conversions after 4 h when a solution of substrates **1** and **2** in DMSO was layered on top of the gel (entry 6). However, an increase of the surface area by mechanical fragmentation of the gel body into smaller pieces afforded the desired β-nitroalcohol **3a** in 83% yield (entry 7). Finally, the incorporation of the substrates in neutral ASFS (c = 20 g silk L^{-1}) caused precipitation of the protein, and **3a** was obtained in only 22% yield (entry 8). Null conversions in control experiments performed in the absence of SF material (entries 2 and 5) confirmed its catalytic role in the nitroaldol reaction.

An interesting correlation was found between the reaction outcome (Table 1) and the morphology of the SF materials observed by scanning electron microscopy (Figure 2). The

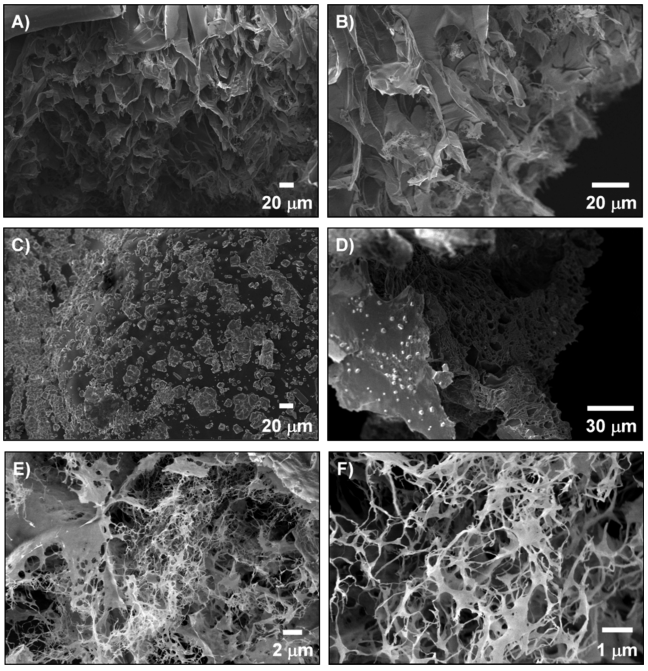
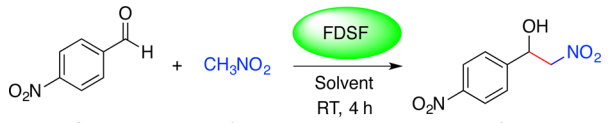


Figure 2. FE-SEM images of different SF-based materials depicted in Table 1: (A) FDSF (magnification 285×), (B) FDSF (magnification 676×), (C) MPSF (magnification 246×), (D) MPSF (magnification 554×), (E) xerogel obtained from SFHG (magnification 3.45K×), and (F) xerogel obtained from SFHG (magnification 11.04K×).

most efficient material, FDSF, displayed a highly ordered, macroporous (about 20 μm in diameter), spongy network structure. Such structure could strongly favor the adsorption of small molecules and ions presented in the medium via electrostatic interactions (e.g., physical adsorption), hydrogen bonding, and/or van der Waals forces. However, MPSF showed a closed, amorphous, granular shell that covered a core porous structure with smaller pore diameters (about 1.0−10 μm) than those observed in FDSF (Figure 2C,D). Interestingly, a highly porous and fibrilar structure was also observed for SFHG (pore diameter about 0.2−1.0 μm) (Figure 2E,F), in which acceptable conversions were obtained upon fragmentation of the bulk gel. These results suggest a complex mechanism involving both diffusion-controlled processes and surface reactivity.

The facile preparation of FDSF and its better performance in the catalyst screening made us use this material for further studies. Table 2 outlines the results of solvent screening for the

Table 2. Solvent Screening for FDSF-Mediated Nitroaldol (Henry) Reaction[a]



1a 2 3a

entry	solvent	yield **3a** (%)[b]
1	DMSO	95
2	DMSO	0 (control)[c]
3	DMF	36
4	DMF	2 (control)[c]
5	water	31
6	water	0 (control)[c]
7	EtOH	<2
8	Et_2O, CH_3CN, CH_2Cl_2, THF, toluene	0

[a]Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 0.1 mmol), nitromethane (**2**, 1.0 mmol), FDSF (10 mg), solvent (0.5 mL), 4 h, room temperature. [b]Determined by ^{1}H NMR analysis of the crude product based on aldehyde proton. Reported yields correspond to the average values of two independent experiments (STDV = ± 2). [c]Control experiment performed in the absence of FDSF.

nitroaldol reaction mediated by FDSF. In agreement with our previous observations, the best results were achieved in DMSO (entry 1), whereas modest yields were obtained in DMF (entry 3) and H_2O (entry 5). In contrast, only traces of the product could be detected when the reaction was carried out in EtOH (entry 7), and no conversion was observed in Et_2O, MeCN, CH_2Cl_2, THF, and toluene (entry 8).

Additional control experiments were also carried out in order to rule out any catalytic effect of possible impurities (e.g., Li^+ ions), which could be leached from the FDSF during the reaction. For these experiments, the FDSF material was matured in DMSO in the absence of the substrates **1** and **2**. After 4 h, the supernatant solution was separated from the solid catalyst. The solution was combined with **1** and **2** (mixture I), and the catalyst was mixed with 0.5 mL of DMSO containing **1** and **2** (mixture II). The molar ratio of the substrates and total concentration in each case were the same than those used in standard experiments. Both reaction mixtures were stirred for additional 4 h. Nitroaldol product **3a** was obtained in only 2% yield using mixture I, whereas mixture II afforded the desired product **3a** in 88% yield. These results confirm the heterogeneous nature of the process and the negligible catalytic effect caused by possible leaching of Li^+ ions. In order to quantify the possible effect of Li^+ ions if they were accessible, the exact amount of Li^+ ions in the FDSF material was estimated by ICP-OES in 0.027 mmol per mg of FDSF. Thus, an equivalent LiBr stock solution was prepared in DMSO and used to carry out the model nitroaldol reaction in homogeneous conditions. In this case, the conversion toward the desired β-nitroalcohol **3a** was only 15%.[39−42] In addition, another control experiment was carried out using silk sericin protein instead of FDSF. The silk sericin content lies between 17% and 30% in cocoons of *Bombyx mori*,[43−45] which is usually

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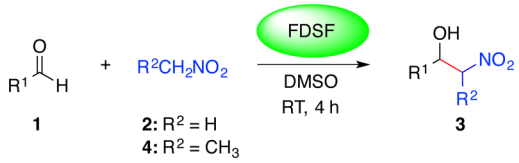
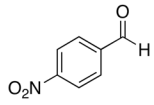
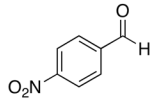
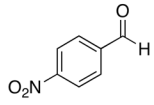
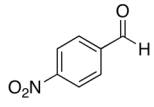
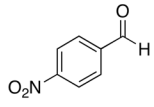
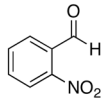
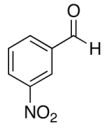
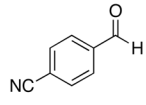
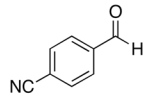
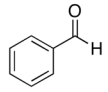
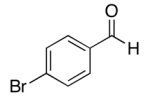
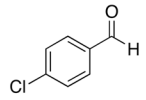
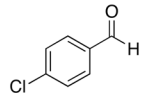
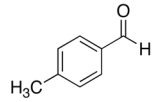
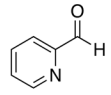
Table 3. Substrate Scope of the FDSF-Mediated Nitroaldol Reaction in DMSO[a]



1 2: R^2 = H 4: R^2 = CH_3 3

entry	R^1CHO	R^2	yield **3** (%)[b]	dr[c]
1		H	95[d]	NA
2		H	80[e]	NA
3		H	0 (control)[f]	NA
4		CH_3	94	1:1.1
5		CH_3	8 (control)[f]	1.4:1
6		H	90	NA
7		H	97	NA
8		H	91	NA
9		CH_3	92	1:1.3
10		H	43	NA
11		H	88	NA
12		H	59	NA
13		CH_3	87	1.1:1
14		H	11	NA
15		H	82	NA

[a]Reaction conditions: Aldehyde (**1**, 0.1 mmol), nitroalkane (**2** or **4**, 1.0 mmol), FDSF (batch 1, 10 mg), DMSO (0.5 mL), 4 h, room temperature. [b]Determined by ^{1}H NMR analysis of the crude product based on aldehyde proton. Reported yields correspond to the average values of two independent experiments (STDV = ± 2). [c]Diastereomeric ratio (*anti*/*syn*) determined by ^{1}H NMR analysis (NA = not applicable). [d]FDSF was obtained by freeze-drying about 20 mL of ASFS (3.7 wt %) in a 100 mL flask. [e]FDSF was obtained by freeze-drying about 2 mL of ASFS (5.4 wt %) in a 5 mL vial. [f]Control experiment performed in the absence of FDSF.

separated from the silk fibroin during the degumming process in $NaHCO_3$ solution. The use of pure silk sericin afforded a considerable lower conversion toward **3a** (20%). All results from the control experiments suggest that mainly the silk fibroin protein is responsible for the catalytic activity in the nitroaldol reaction.

With these results in hand, we further evaluated the substrate scope of FDSF in DMSO (Table 3). In general, aromatic aldehydes with strong electron-withdrawing groups could be converted exclusively to the corresponding *β*-nitroalcohol in very good yields between 90% and 95% (entries 1, 4, 6–8). No major effects of the position of the substituents in **1** were observed (entries 1, 6, 7). To ensure reproducibility of the process, different batches of FDSF were randomly tested for the reaction between **1a** and **2** resulting in only slight variations in the yield of **3a** within 80% and 95%. It should be noted that the preparation of FDSF at different scales afforded materials with different surface areas, which could explain the variation in reactivity. In general, less spongy and fluffy material was obtained in low-scale preparations (entries 1 and 2). Halogenated aromatic aldehydes such as 4-bromobenzaldehyde (entry 11) and 4-chlorobenzaldehyde (entry 12) could be converted into the desired product in good (88%) and moderate yields (59%), respectively. The *β*-nitroalcohol product derived from benzaldehyde (entry 10) could be also obtained in moderate yield (43%), whereas the use of aromatic aldehydes with electron-donating groups such as 4-methylbenzaldehyde (entry 14) gave poor yield (11%). Interestingly, the heteroaromatic aldehyde 2-pyridinecarboxaldehyde (entry 15) could be also transformed in good yield (82%). Very high conversions (ca. 88–100%) were also observed within 14–24 h for other related aldehydes such as 5-bromo salicylaldehyde, 4-bromo benzaldehyde, or 4-fluoro benzaldehyde (data not shown). In contrast, aliphatic aldehydes could not convert into the desired product. When nitroethane (**4**) was used as nucleophile in combination with **1a**, no significant differences in comparison to nitromethane were observed (entries 4, 9, 13). Thus, both the size of the nucleophilic carbanion and its

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pK_a (pK_a values:[46] **2** = 10.2; **4** = 8.6) showed in this case a minor effect on the reaction outcome. In comparison to other standard catalysts, the performance of FDSF was, for example, similar to the catalysis by Et_3N in water (see Supporting Information).

It is worth mentioning that negligible enantioselectivity (ee ≤1%)[47−49] and low diastereoselectivities (*anti/syn* ratio) were routinely observed. On the basis of the early reports of Evans and co-workers reporting an asymmetric variant of the Henry reaction catalyzed by a chiral copper(II) complex,[50] we also tested Cu(II)-doped FDSF materials.[51] However, this variation also yielded the racemic mixture of the desired nitroaldol product.

In terms of reusability, FDSF showed a remarkable deactivation after the first almost quantitative cycle (Figure 3). Although the exact deactivation mechanism during the nitroaldol (Henry) reaction remains unclear, blocking of the catalytic sites by chemical poisoning of the surface seems to be crucial. As we have described earlier with other proteins,[2,3] the potential chemical evolution of intermediate imines (e.g., via reductive Cannizaro-like processes or formation of cross-linked aminals) and excess of nitromethane that could undergo partial association with free amine and hydroxyl groups, as well as slow reaction kinetics, may contribute to form an inactive coat blocking the access to the basic catalytic sites of the protein. In agreement, FE-SEM imaging of FDSF before and after the nitroaldol reaction showed a significant morphological change from a porous and spongy-like structure to a very clotted surface (inset Figure 3).

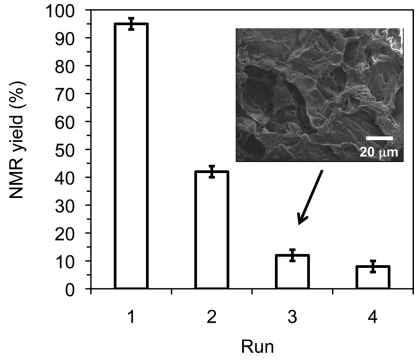


Figure 3. Recycling study of the FDSF-mediated Henry reaction. Inset: FE-SEM image of the clustered surface structure of FDSF after the third run in the nitroaldol reaction (magnification 634×). Attempts to reactivate FDSF by alternative washing with different solvents after the work-up were fruitless.

Knoevenagel Reaction. The potential catalytic effect of FDSF in a model Knoevenagel condensation was also studied. This reaction was carried out between 2-nitrobenzaldehyde (**5**) and ethyl cyanoacetate (**6**) in DMSO at room temperature. As shown in Figure 4, the reaction was completed after 3 h yielding the desired product **7** in 84% (only the *E*-isomer was detected, and no self-condensation or Cannizaro products were observed). The control reaction between **5** and **6** carried out in the absence FDSF showed only traces of product **7**. Deactivation of the FDSF catalyst after the Knoevenagel condensation was also observed, albeit this process was significantly slower in comparison to that in the nitroaldol reaction (i.e., yield of **7**: 84% (1st run), 60% (2nd run), 34% (3rd run), 20% (4th run)).

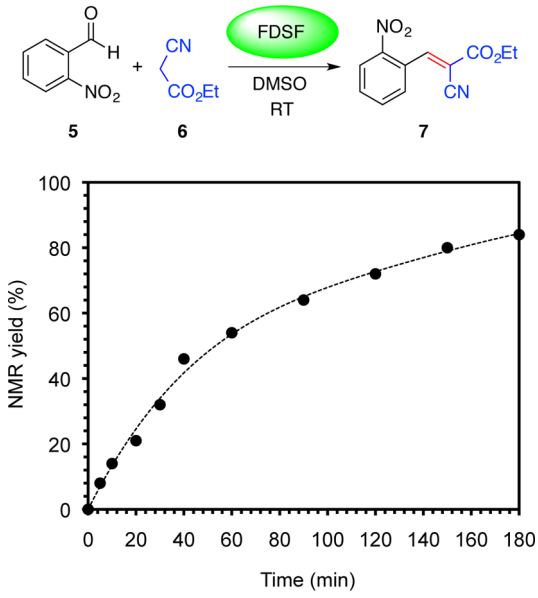
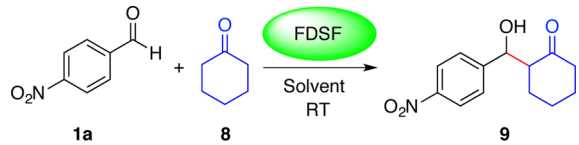


Figure 4. Kinetics of the Knoevenagel model reaction between 2-nitrobenzaldehyde (**5**) and ethyl cyanoacetate (**6**) mediated by FDSF. A comparison with other catalysts is given in the Supporting Information.

Direct Aldol Reaction. Despite the efficiency of FDSF in both nitroaldol and Knoevenagel reactions, no activity was observed for the direct aldol reaction between acceptor **1** and cyclohexanone (**8**) as donor in different solvents (i.e., DMSO, DMF, water, THF) for 48 h at room temperature (Table 4, entries 1, 5, 9). By increasing the temperature to 37 °C, the aldol product **9** was obtained in 8% yield after 48 h both in DMSO (entry 2). Extension of the reaction time to 7 days provided only a slight increase in the yield (entry 3). The use of water instead of DMSO caused a considerable background

Table 4. Solvent Scope of the FDSF-Mediated Aldol Reaction[a]



1a **8** **9**

entry	solvent	temperature (°C)	time (days)	yield **9** (%)[b]
1	DMSO	RT	2	0
2	DMSO	37	2	8[c]
3	DMSO	37	7	10[c]
4	DMSO	37	7	<1 (control)[d]
5	DMF	RT	2	<1
6	H_2O	RT	2	2
7	H_2O	37	7	13[c]
8	H_2O	37	7	7 (control)[d]
9	THF	RT	2	0

[a]Reaction conditions: 4-Nitrobenzaldehyde (**1a**, 0.1 mmol), cyclohexanone (**8**, 2.0 mmol), FDSF (10 mg), solvent (0.5 mL). RT = room temperature. The molar ratio aldehydes:ketone 1:20 was used to minimize self-condensation of the acceptor and favor cross-condensation. [b]Determined by ^{1}H NMR analysis of the crude product. [c]Approximate diastereomeric ratio *anti/syn* = 1:1 (for the reaction in DMSO), 1:3 (for the reaction in H_2O). [d]Control experiment performed in the absence of FDSF.

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reaction in the absence of FDSF (entries 6–8). Only minor enantioselectivity was observed for the reaction carried out in water (entry 7) (i.e., 6% ee of the major *anti* isomer; 4% ee of the minor *syn* isomer).

CONCLUSIONS

In conclusion, this study has demonstrated an intrinsic ability of silk fibroin-based materials, derived from the cocoons of the silkworm (*Bombyx mori*), to promote C–C bond formation under mild conditions (i.e., DMSO or H_2O at RT). Among different materials, FDSF was found to be the most active material in the nitroaldol (Henry) reaction and Knoevenagel condensation followed by MPSF and SFHG. In general, aromatic aldehydes with strong electron-withdrawing groups showed the best yields. In sharp contrast, poor activity was observed in the direct aldol reaction, albeit with slight enantioselectivity in some cases. Although the protein catalyst could be reused in further runs, progressive deactivation was observed especially in the nitroaldol reaction, presumably due to the formation of inactive surface coating. Overall, the results confirm that the morphology and/or physical state of the silk fibroin plays a significant role in the kinetics when mediating these types of reactions. Studies toward other forms of silk-based materials, including silk-based nanoparticles, their reusability, and their potential influence in different chemical processes are currently underway in our laboratories.[52]

ASSOCIATED CONTENT

Supporting Information

Selected FT-IR spectra, TGA plots, ^{1}H NMR spectra, HPLC chromatograms, and mechanistic considerations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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