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Production of silk fibroin nanoparticles using ionic liquids and high-power ultrasounds

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ABSTRACT: Biopolymeric nanoparticles have attracted great research interest in the last few years due to their multiple applications. This article describes how high-power ultrasounds are capable of enhancing the dissolution process of silk proteins in ionic liquids (ILs) and how silk fibroin nanoparticles (SFNs) can be obtained directly from the silk/ionic liquid solution (SIL) by rapid desolvation in polar organic solvents. The silk fibroin integrity is highly preserved during the dissolution process, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the SIL. These regenerated SFNs are insoluble in water and other common organic solvents and are indistinguishable from the classical SFNs with respect to their diameter (180 ± 5 nm), Zeta potential (-25 ± 3 mV), high degree of β-sheet and low cytotoxicity. Large amounts of silk can be turned into biomaterials directly from the SIL solution for use in a wide range of applications, while the ILs can be recovered from the coagulant solution under reduced pressure and reused without loss of their solvent properties. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41702.

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INTRODUCTION

Owing to its excellent biocompatibility and mechanical properties, silk is uses as an attractive biomaterial in biomedical and tissue engineering applications.[1] Silk fibroin (SF) obtained from *Bombyx mori* cocoons has a unique combination of mechanical and biological properties, including nontoxicity, biocompatibility, and biodegradability. This biomaterial, formulated as particles, has potential applications in medicine for its capacity to adsorb, transport, and deliver a wide range of bioactive molecules.[2] Silks are insoluble in most solvents, including water, dilute acid, and alkali. The enhanced stability of silk biomaterials is attributed to their extensive hydrogen bonding, the hydrophobic nature of much of the protein, and a significant degree of crystallinity.[3]

In tradition, the following two solvent systems have been used to dissolve degummed silk fibers: ionic aqueous solutions, such as 9.3*M* LiBr or 50 wt % $CaCl_2$ solution,[4] and ionic hydroalcoholic solutions, such as a $CaCl_2$/ethanol/water mixture (Ajisawa's reagent).[5] These solutions require extensive dialysis in deionized water before being concentrated to the required level. However, the processes of both dialysis and concentration are time-consuming and the solutions are unstable, with a short half-life before the protein becomes unstable and aggregates to a gel state. But without postdrawing, the fibres[6,7] and films[8] produced from these regenerated SF aqueous solutions are generally brittle. Alternatively, for long-term storage, aqueous solutions of SF can be lyophilized and redissolved in organic solvents such as 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). However, these solvents are toxic and extremely corrosive, requiring considerable care in handling.[9]

The degradation of fibroin molecules caused by high-temperature dissolution in inorganic salt systems is the origin of the poor mechanical properties of SF-based biomaterials.[10,11] Previously, silks were dissolved in ionic liquids (ILs) such as

Additional Supporting Information may be found in the online version of this article.

1-butyl-3-methylimidazolium chloride ([bmim$^+$][Cl$^-$]), to form a stable SF solution.[12–16] Silkworm SF solubility in ILs is attributed to the ability of the anion (mainly halogens or small carboxylates) to disrupt the hydrogen bonds in the SF β-sheets.[17] The main advantage of using ILs as solvents is that the total number of steps required for the dissolution process is reduced, as the cocoon can be dissolved directly in the selected ILs.

The use of ILs for silk dissolution is also a considerably greener option compared with using HFIP, a volatile, corrosive, and toxic solvent, as it presents unnecessary pollution.[18] But complete dissolution of silk proteins using the above-described methods takes several hours, even with intense heating at 100°C,[12] resulting in the loss of protein integrity. Long treatments lead to breakage of the peptidic chains and poor mechanical properties of the resulting biomaterials. The silk/ionic liquid (SIL) solution is highly viscous and this viscosity increases at high protein concentrations, which is a drawback for industrial application. For this reason, the process of silk dissolution was improved by applying high-power ultrasounds to the SIL mixture—to accelerate the process, thus avoiding long heating treatments—and by adding water to reduce the viscosity.

High-power ultrasounds have two important synergistic effects on the mixture: rapid heating and efficient disruption of the fibers at the same time. The ultrasonication rapidly raises the temperature of ILs and helps to introduce the ILs into cohesively bound SF threads and disrupt the hydrogen bonds until their complete dissolution. ILs have also proved their applicability as solvents for other biopolymers, such as cellulose. Swatlosky *et al.*[19] reported that the method used to prepare the solution greatly affected the solubility. In particular, these authors used microwave heating on a [bmim$^+$][Cl$^-$]/cellulose mixture to dissolve 25% (w/w) cellulose and compared the results with those obtained for a 10% (w/w) cellulose solution heated in an oil bath at 100°C. However, the peak temperature reached during the microwave dissolution step was not reported and neither did the authors analyze the extent of cellulose degradation. Novel composites can also be prepared by mixing different polymer solutions in ILs, such as cellulose, polyvinyl acetate (PVA) or polyurethane, and SF.[20]

Ultrasonication has been applied to dissolve cellulose in ILs and was capable of enhancing the dissolution process of water-insoluble biopolymers with a high number of hydrogen bonds.[21] To date, ultrasonication has only been applied to an aqueous SF solution, with the aim of promoting gelation of the biopolymer.[9] Although SF dissolved in ILs has been regenerated in films, sponges, and fibres,[12–15] particle coagulation from the IL-dissolved SF has not been reported.

By applying the precipitation method commonly used for the coagulation of an aqueous SF solution in a water-miscible organic solvent, it is possible to obtain particles of regenerated SF.[22] Previous data suggest that silk films obtained from an ionic liquid solution have no detrimental effect on cell viability, differentiation or gene expression, and the use of ILs as a solvent for SF dissolution provides an avenue for the fabrication of silk scaffolds for tissue engineering applications.[14] Regenerated SFNs obtained from coagulation of the SIL solution in methanol should show a similar lack of effect on cell viability, although further experiments are necessary to confirm this.

The first part of the study is dedicated to the comparison of the classical dissolution process, using an oil bath as heat source, with high-power ultrasounds and to the characterization of SILs mixtures. The SF integrity is highly preserved during the dissolution process, as confirmed by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) of the SIL and HPLC/MS of the selected bands. The second part is dedicated to the preparation of SFNs directly from the SIL solution, by rapid desolvation in polar organic solvents. These new SFNs are insoluble in water and other common organic solvents and are indistinguishable from the classical SFNs regarding their diameter, Zeta potential, high degree of β-sheet and low cytotoxicity. The ILs can be recovered from the coagulant solution under reduced pressure and reused without loss of their solvent properties.

EXPERIMENTAL

Material

White silk cocoons (SC) were obtained from silkworms of *B. mori* reared in the sericulture facilities of the IMIDA (Murcia, Spain) and raised on a diet of natural *Morus alba L.* fresh leaves. The intact pupae were extracted from the cocoons two to seven days after spinning by cutting open the cocoons. This method avoids possible contamination and thermal degradation from the industrial process of baking the pupae in the cocoons. Degummed silk (SF) was used for the solubility test. Briefly, raw white silk cocoons (SC) were boiled twice in a 0.05*M* Na_2CO_3 aqueous solution for 45 minutes. The remaining SF was rinsed thoroughly with ultrapure water and dried prior to use. In the dissolution experiments using ultrasonication, the SC and SF were cut into small pieces (~100 mg) and added to the ILs. All the ILs (97% purity) were purchased from IoliTec GmbH (Germany) and were used without further purification. Purified water (18.2*M*Ωcm at 25°C; from a Millipore Direct-Q1 ultrapure water system, Billerica, MA) was used throughout. All other chemicals and solvents were of analytical grade and were used without further purification.

Test of the Silk Fibroin Solubility in ILs

To test SF solubility, dissolution experiments were conducted in an open glass vessel. To determine the solubility of the SF in the selected ILs, protein was added slowly to the melted ionic liquid while providing magnetic agitation. The temperature of the mixture was maintained at 90°C with a temperature-controlled oil bath. The resultant SIL solutions were clear and amber in color, and above 10% (w/w) -were quite viscous.

Dissolution of Silk Proteins in ILs Using Pulsed Ultrasonication

Small pieces of ~100 mg of silk cocoon or degummed SF were added sequentially to 5 g of molten IL, in a vessel resistant to high-temperature and high-power ultrasounds. After each addition, the mixture was treated on a Sonifier Branson 450D (Emmerson Ultrasonic Corporation. Dansbury, USA) subjected to a pulsating ultrasonication steps at 30% amplitude, with a 3/

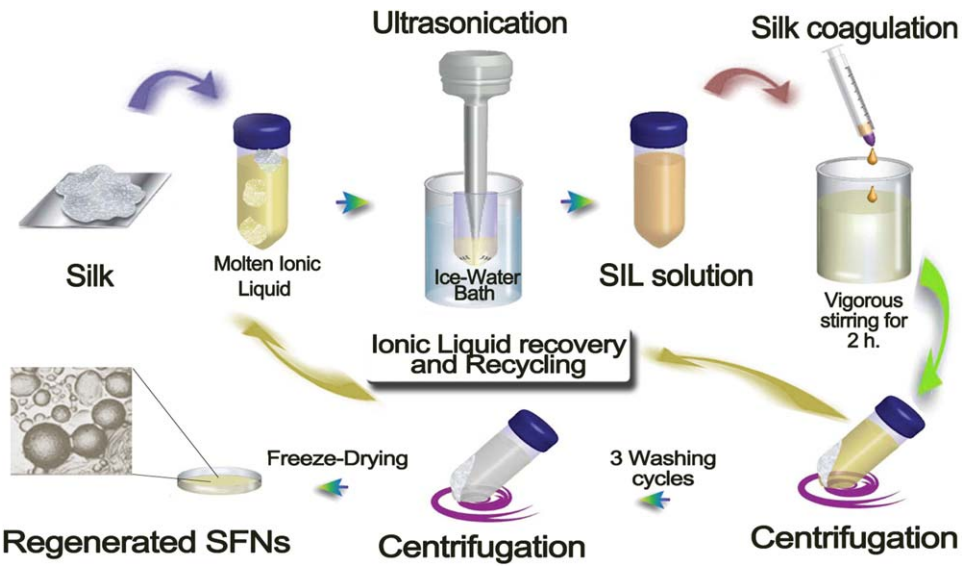

Figure 1. Scheme of the overall process of SF dissolution, using ILs and ultrasonication, and consequent SFNs preparation from SIL solutions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

8″ tapered horn and temperature automatically limited to 100″C (Supporting Information, Figure S1), until complete dissolution. Complete dissolution of silk fibers was confirmed by microscopy, using a Nikon Eclipse 50*i* microscope (equipped with a CCD camera DS-Fi1) and NIS-Elements Ver. 2.0 software.

Characterization of SIL Solutions

Silk protein integrity in the SIL solutions was confirmed by SDS-PAGE, followed by trypsin digestion and HPLC-MS identification of the protein fragments in selected bands. The SDS-PAGE was performed according to the Laemmli protocol, with a 4–20% gradient acrylamide gel (Amersham GE-HC).[23] The setup used was a horizontal Gel-Box electrophoresis chamber (Amersham GE-HC). After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue (Acros Organics, Belgium). Two molecular weight markers were loaded: ColorBurst™ Electrophoresis Marker (SIGMA-ALDRICH, St. Louis, MO) and BenchMark™ protein ladder (Invitrogen). Protein concentrations in the samples were unified at 50 μg per lane by diluting the different ionic liquid solutions of SF with MilliQ water. These samples were loaded under denaturing conditions by adding β-mercaptoethanol (10% v/v) to the loading buffer and heating at 100″C for 5 minutes immediately before electrophoresis.

Selected gel bands were cut and digested with Trypsin Gold (Promega), according to the manufacturer's protocol for mass spectrometry analysis. Using Proteome Discoverer Ver. 1.3 software and searching in the SwissProt Data Bank (2011),[24] the searching tolerance was set to $\pm$ 1.5 Da for precursors and $\pm$ 0.8 Da for fragments. A filter for FDR $P < 0.01$ was established and at least two unique peptides per protein.

Preparation of Silk Fibroin Nanoparticles

Figure 1 summarizes the overall process of SFNs preparation from SIL solutions. The preparation of SFNs was based on the method described previously by Zhang *et al.*, with modifications.[22]

Briefly, to the freshly prepared SIL solution, ultrapure water (MilliQ, 18.2 $M\Omega$·cm) was slowly added to reduce viscosity. After cooling to ~45°C, the SIL solution was dripped (~1 drop every 2 seconds) onto 100 mL of gently stirred cold MeOH. A milky white suspension appeared after a few drops, and the suspension was allowed to reach room temperature while stirring for 2 hours. Then, the particle suspension was transferred to centrifuge vials and centrifuged at 18,000 *g* for 15 minutes, at 4°C (Eppendorf Centrifuge 5810R equipped with an F-34-6-38 rotor).

The supernatant—free of particles—was removed, filtered (0.22-µm disposable PTFE filters) and reserved for subsequent recycling of the IL. An equal volume of fresh methanol was added to the vial, and the white precipitate was suspended by vigorous stirring in a vortex mixer for 2 minutes. The centrifugation was repeated under the same conditions. The white precipitate was subjected to successive rinses with ultrapure water in a water/RSF mixture (ratio of 20:1) until no AgCl precipitate was observed after the addition of 0.1 N $AgNO_3$ to the washing water after centrifugation. The particles were lyophilized in an Edwards Modulyo 4K Freeze Dryer for 72 hours, at −55°C and 0.5 mbar, and regenerated SF was obtained as lyophilized dry particles. The methanolic fractions were mixed before recovery of the IL, by removal of the methanol/water on a rotary evaporator at 80°C and 80 mbar. The ILs were kept in a desiccator until reuse.

Characterization of Silk Particles

The particles were characterized by scanning electron microscopy (SEM), dynamic light scattering (DLS), and infrared spectroscopy (FTIR). A morphological examination of the nanoparticles was made using a scanning electron microscope (JEOL-JSM-6100; JEOL-Japan). One drop of nanoparticle suspension was fixed on an aluminum stub, coated with gold (under vacuum, by an auto fine coater), and examined at different magnifications. The mean diameter, size distribution, and

Zeta potential were measured using a Malvern Zetasizer Nano ZS instrument. All measurements were performed in purified water at 25°C or in Dulbecco's Modified Eagle Medium (DMEM) (without Fetal Bovine Serum (FBS) supplementation) at 37°C. The Z-average and polydispersity were obtained with the software provided by the manufacturer. The Z-average diameter and Zeta potential values were calculated from the measurements performed in quintuplicate. The infrared spectra of the pure ionic liquid, SFNs from SIL solutions, and SFNs obtained from the Ajisawa's solvent system[22] were recorded using a Perkin-Elmer Spectrum 100 Series FTIR spectrometer (Norwalk, CT, USA) controlled with Spectrum Software Ver. 6.1.0.0038. Samples (~2 mg) were mixed with ~198 mg of KBr (Sigma-Aldrich) and ground into a fine powder using a mortar and pestle, before being compressed into a disc (Perkin Elmer 15.011, USA). Each spectrum was acquired in transmittance mode (10 scans) with a resolution of 4.0 cm^{-1} and a spectral range of 4000–500 cm^{-1}. The analysis was finally focused in the 2000–500 cm^{-1} range, which is the most informative in the SF spectrum.

Cell Viability/Cytotoxicity Studies

Murine fibroblasts (cell line L929) were chosen for cell culture studies, as these fibroblasts are highly stable, fast growing and commonly used for cytotoxicity experiments. The fibroblasts were seeded onto 48-well tissue culture plates at a density of $3.0 \cdot \times 10^4$ cells/well for 24 hours, after which the growth medium was removed and replaced with the medium containing SFNs obtained from a SIL solution coagulated in methanol. Classical SFNs were obtained by following *Zhang*'s method[22], and growth medium without nanoparticles was used as the control. The culture medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL), at 37°C in a 7.5%-CO_2 atmosphere.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay was used to measure the cell cytotoxicity. L929 murine fibroblasts were plated at a density of $3.0 \cdot \times 10^4$ cells/well in 48-well plates, at 37°C in a 7.5%-CO_2 atmosphere. After 24 hours, the culture medium of each well was replaced with fresh medium containing nanoparticles of selected concentrations (100 and 200 µg/mL). The SFNs prepared with the newly-developed method of silk dissolution in different ILs were used, to analyze their cytotoxicity. After 24 hours, the medium was removed, and 500 µL of MTT (Sigma, St. Louis, MO) dye solution (1 mg/mL in DMEM without phenol red) was added to each well, followed by incubation for 4 hours at 37°C and 7.5% CO_2. Then, the MTT solution was removed, and formazan crystals were solubilized with 200 µL of DMSO per well, vigorously shaking the plates for 5 minutes to dissolve the reacted dye. The absorbance of each well was read on a microplate reader (BMG Fluostar Galaxy) at 570 nm; the reference wavelength was 690 nm. The data are presented as means ± SD (standard deviation), calculated from five samples per condition. The assumptions of normality (Kolmogorov–Smirnov, $P > 0.05$) and homocedasticity (Levene, $P > 0.05$) were met, and the statistical significance was determined using the Tukey ($P < 0.05$) and ANOVA ($P < 0.05$) parametric tests.

Table I. Solubility (%wt) of Silk Fibroin in Selected ILs at 90 °C (heated in an oil bath)

IL	SF solubility (wt %)	IL	SF solubility (wt %)
[mim^+][Cl^-]	S(>12%)	[eim^+][Cl^-]	Is
[$emim^+$][Cl^-]	S(>23%)	[$emim^+$]$EtSO4^-$	Is
[$pmim^+$] [Cl^-]	S(>15%)	[$emim^+$]TfO^-	Is
[$bmim^+$][Cl^-]	S(>12%)	[$bmim^+$]$OctSO4^-$	Is
[$hmim^+$][Cl^-]	S(>11%)	[$bBmim^+$]$PF6^-$	Is
[$omim^+$][Cl^-]	Is	[3^-MEP^+]$EtSO4^-$	Is
[$dmim^+$][Cl^-]	Is	ETAN	Is

Is - Insoluble after 24 h at 90 °C; S – Soluble

RESULTS AND DISCUSSION

Test of the Solubility of Silk Fibroin in ILs

The dissolution of *Bombyx mori* SF using an oil bath as the heat source was investigated in the assembled state of the fibres. Table I summarizes the results. Dissolution of the SF samples was confirmed by observation of fiber dissolution under an optical microscope. Complete dissolution took over 1 hour at 100°C, as described by Phillips *et al.*[12] The saturated solubilities by weight for SF in 1-alkyl-3-methylimidazolium chlorides are dependent on the length of the alkyl substituent of the imidazolium ring, as can be seen in Table I. Of the chlorides, [$emim^+$][Cl^-] showed the highest solvent capacity, reaching 25% (w/w). These results are concordant with those described previously in the literature.[12]

The results confirm that 1-alkyl-3-methyl imidazolium chlorides act as good solvents for silk dissolution when the length of the alkyl chain is less than eight carbons.[12] The disruption of protein hydrogen bonds requires mid-high polarity and greater lengths increase the hydrophobicity of the organic cation, so that long-aliphatic-chain ILs cannot act as solvents for SF. In the same way, when ILs have highly-hydrophobic anions, they are not able to dissolve SF.

Dissolution of Silk Proteins Using Ultrasonication

To test the effect of ultrasonication on the silk fibers, the ILs with the greatest ability to act as solvents for SF were selected. Thus, silk proteins were also successfully dissolved in the 1-alkyl-3-methylimidazolium chlorides (where 1-*alkyl* is: *methyl* [mim^+][Cl^-]; *ethyl* [$emim^+$][Cl^-]; *propyl* [$pmim^+$][Cl^-]; *butyl* [$bmim^+$][Cl^-] or *hexyl* [$hmim^+$][Cl^-]), using pulsed ultrasonic treatment to heat the solution efficiently and achieve fast disruption of the finers. The saturated solubility by weight and the time required for silk dissolution in selected ILs are listed in Table II.

While the classical heating method needed hours to complete the dissolution, the application of ultrasounds to the mixture achieved a significant reduction in the time necessary to complete the SF dissolution process in ILs at 100°C. The solvent effect of the cations and anions of the ILs that break the β-sheet hydrogen bonds network was enhanced by the

Table II. Solubility and Time Required for the Dissolution of Silk Proteins, in Selected ILs

	Silk fibroin (SF)		Silk cocoon (SC)	
Solvent	Solubility (%wt)	Time (min.)	Solubility (%wt)	Time (min.)
[mim$^+$][Cl$^-$]	12.5 ± 0.1	4	12.5 ± 0.1	17
[emim$^+$][Cl$^-$]	23.0 ± 0.3	17	18.7 ± 0.6	67
[pmim$^+$][Cl$^-$]	15.2 ± 0.3	14	17.6 ± 0.1	27
[bmim$^+$][Cl$^-$]	12.7 ± 0.6	5	12.9 ± 0.4	24
[hmim$^+$][Cl$^-$]	10.9 ± 0.2	8	11.1 ± 0.3	20

Solubility is presented as the average value ± SD (standard deviation) of three independent experiments.

ultrasounds. The ILs with greater solvent ability took longer to reach saturation due to the increase in viscosity of the SIL solution . After recycling, ILs can be used without the loss of their solvent properties in at least four successive processes

Although the solubility of SF was highest in [emim$^+$][Cl$^-$], for particle formation, it is not necessary to reach a concentration higher than 10% (w/w), since increased viscosity represents a handicap for handling. For SFNs preparation, 10% (w/w) SIL solutions were prepared by following the above-mentioned procedure.

Characterization of SIL Solutions

In the SDS-PAGE experiments, it was found that shorter protein dissolution times at high temperatures prevented the high degree of peptidic chain fragmentation that occurs with the dissolution methods commonly used. As can be observed in Figure 2, the molecular masses of the fragments of the SIL solutions are almost identical to that of the SF present in the silkworm gland.[25]

The peptide distribution in the polyacrylamide gel shows discrete bands that were identified by HPLC/MS as highly conserved SF heavy chain (390.1 KDa), SF light chain (27.7 KDa), and fibrohexamerin P25 (25.1 KDa) in the degummed SF solution. The silk protein solution prepared by dissolution of whole cocoons with ILs and ultrasonication showed the above-mentioned SF peptides and also peptidic chains of sericin 1 (119.1 KDa).[26] The heavy chain of SF is composed of 5263

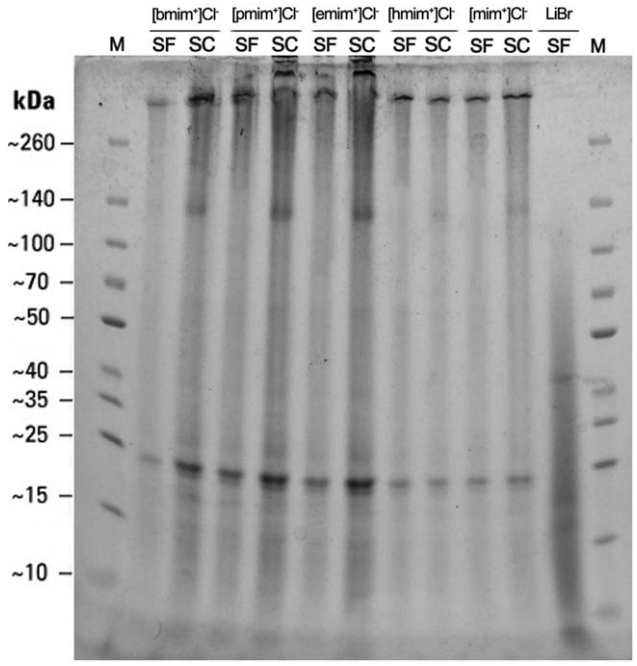

Figure 2. SDS-PAGE analysis of the protein components of silk fibroin (SF) and white silk cocoons (WC) after ultrasonication in the selected ionic liquids: [bmim$^+$][Cl$^-$], [pmim$^+$][Cl$^-$], [emim$^+$][Cl$^-$], [hmim$^+$][Cl$^-$], and [mim$^+$][Cl$^-$]. Lane M: Spectra Multicolor Protein Ladder 10–260 KDa (Thermo), (4–20% gradient Gel Amersham GE-HC).

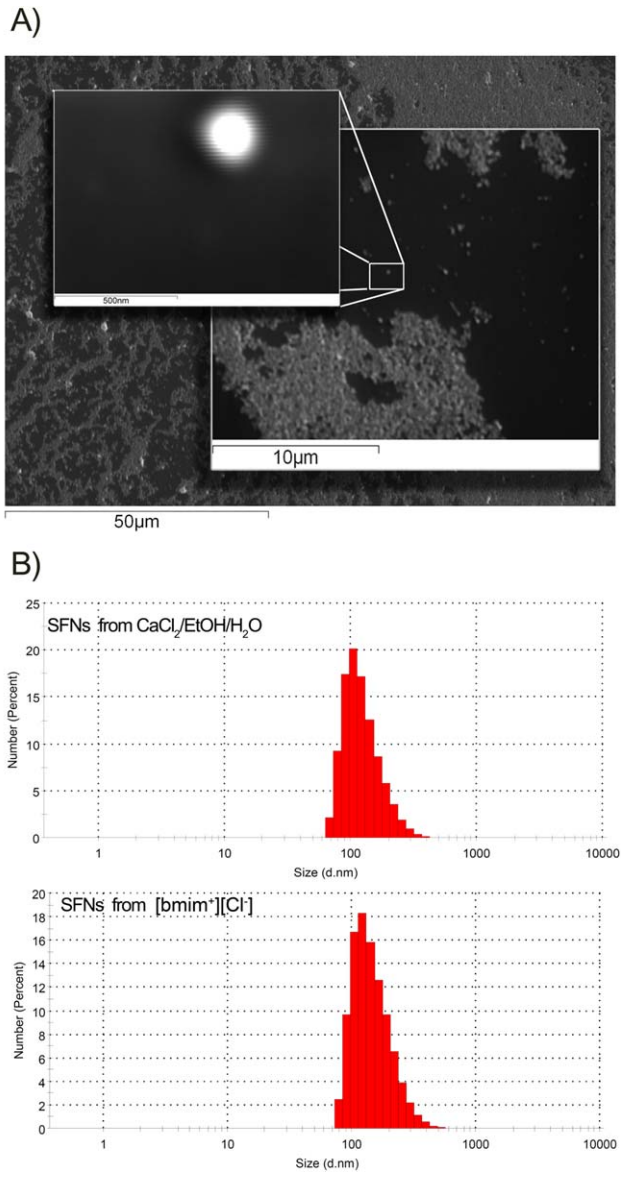

Figure 3. (A) Sequence of SEM images of SFNs regenerated from SF/[bmim$^+$][Cl$^-$] solution (Sequential scale bars: 50 µm, 10 µm, and 500 nm). (B) Comparative size distributions of classical SFNs regenerated from the Ajisawa solvent system[22] and from SF/[bmim$^+$][Cl$^-$] solution (Malvern Zetasizer ZS). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table III. Comparative Values for the Particle Size (diameter), Polydispersity (PdI), and Zeta Potential of Classical SFNs[22] Obtained from $CaCl_2$/EtOH/H_2O Solvent and SFNs Produced from SIL Solutions

	MilliQ water at 25°C			DMEM[c] (without FBS) at 37°C		
Solvent used	Diameter[a] (nm)	PdI[b]	Zpot[a] (mV)	Diameter[a] (nm)	PdI[b]	Zpot[a] (mV)
$CaCl_2$/EtOH/H_2O	174 ± 2	0.121	−26.23 ± 0.59	183 ± 3	0.140	−12.02 ± 0.42
[mim^+][Cl^-]	177 ± 4	0.153	−27.15 ± 0.74	208 ± 4	0.115	−12.08 ± 1.50
[$emim^+$][Cl^-]	181 ± 3	0.230	−25.65 ± 0.90	341 ± 9	0.393	−12.00 ± 2.12
[$pmim^+$][Cl^-]	175 ± 4	0.129	−27.53 ± 0.66	211 ± 4	0.076	−12.22± 1.09
[$bmim^+$][Cl^-]	184 ± 5	0.212	−24.53 ± 1.42	235 ± 4	0.245	−12.24 ± 1.66
[$hmim^+$][Cl^-]	176 ± 3	0.140	−27.90 ± 0.82	200 ± 3	0.133	−11.28 ± 0.55

[a] Z-average ± SD (n = 5) and accumulation times = 100.
[b] Average value.
[c] Dulbecco's modified Eagle's medium

aminoacidic residues, and it is very sensitive to thermal treatment and the solvent system composition.[11] As can be seen in Figure 2. (Lane LiBr SF), the classical methods of SF dissolution in hydroalcoholic/salts solvent systems showed an SDS-PAGE with a continuous smear of fragments of multiple sizes—in contrast with the new, short-time dissolution method which conserved the integrity of the proteins.[11]

Preparation of Silk Fibroin Nanoparticles

The scheme of Figure 1 summarizes the overall process of SFNs preparation from SIL solutions. When a SIL solution is rapidly poured into an excess of a polar organic solvent, such as methanol, ethanol, or acetone, a milky white suspension of silk protein is formed immediately. The SF regeneration in the form of particles was achieved by pouring SIL solution into an excess of methanol. The organic solvent dissolves the IL, and the protein chains are able to reconstitute the hydrogen bonds network and change from a random coil structure to the highly ordered and solid β-sheet conformation in the particles.[22] The viscosity of the mixture also played an important role in nanoparticle formation.

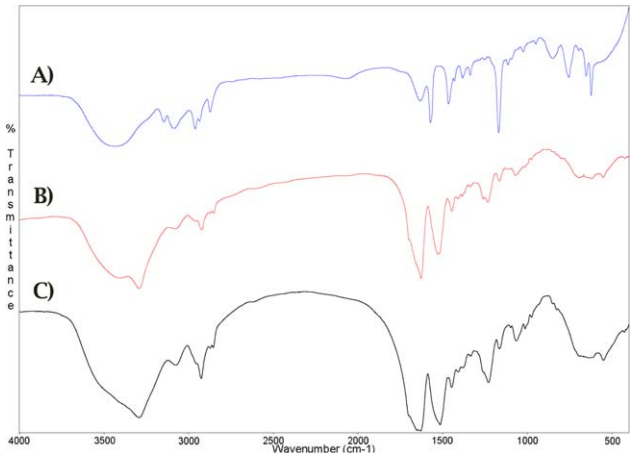

Figure 4. Comparative FTIR spectra of (A) Only [$bmim^+$][Cl^-]; (B) SFNs regenerated from SF/[$bmim^+$][Cl^-] solution; (C) SFNs regenerated from classical $CaCl_2$/EtOH/H_2O solution,[22] measured in a Perkin Elmer 100 Series Spectrometer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Lower the viscosity of the SIL, the more efficient the mixing, and the desolvation of the peptidic chains was faster, leading to the efficient formation of nanoparticles when the SIL viscosity was below 0.5 Pas. Higher viscosities induced poor mixing of the precipitating solvent and the protein solution, leading to the formation of aggregates of micrometric to millimetric size.

The SFNs formation after coagulation of the SIL mixtures in these solvents was an efficient process, with a protein recovery higher than 85% after freeze drying. Most of the ~15% by weight of nanoparticles was lost in the washing process because the smaller particles needed higher *g*-forces to promote their sedimentation in water. But this washing process is essential, to prevent the ILs from being retained in the SFNs and affecting the biomedical application of the particles. The FTIR analysis of the SFNs obtained discounted the presence of ILs in the particles.

After protein precipitation and separation from the coagulant solvent by centrifugation and filtration through a 0.22-μm PTFE filter, to eliminate protein particles, the ILs were recovered with high yields, in excess of 98%, by rotoevaporation under vacuum—because of their negligible vapor pressure.[18] The stability of the ILs was checked routinely by NMR analysis in deuterated methanol—which showed no significant differences from the commercial ILs (data not shown). The ILs were reused, with no decrease in their solvent properties—at least in four process.

Characterization of Silk Fibroin Nanoparticles

Scanning electronic microscopy (SEM) showed that the silk particles were globular granules of a quite-homogeneous size (Figure 3). The stability of the particles was also tested in DMEM culture media. In MilliQ water at 25°C, and the SF particles had an average size of 170–184 nm (Zsizer ZS, Malvern). The results indicate that the particles were slightly larger (183–341 nm) when dispersed in DMEM (Table III); these values are almost identical to those described previously in the corresponding literature.[22,28,29]

The polydispersity values of the silk nanoparticles were similar in all cases, demonstrating that the particles were well dispersed in the medium, with no aggregation. The zeta potential range of the SF particles varied from −25.6 to −27.9 mV in water

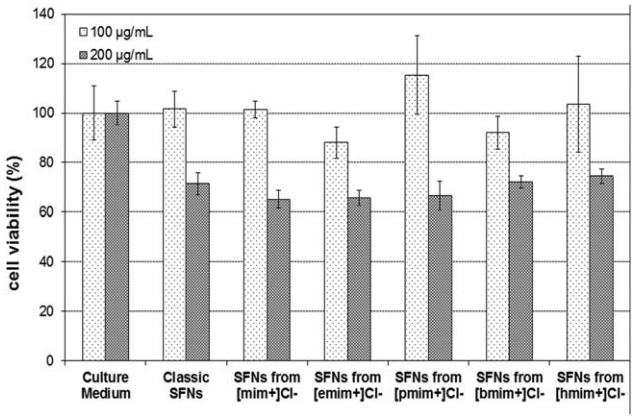

Figure 5. Cell viability (%) of murine L929 mouse fibroblasts after 24 hours of incubation with SFNs (classical and obtained from SIL solutions) relative to the control cells without nanoparticles, as determined by the MTT assay (mean ± SD of n = 6).

and from −11.2 to −12.2 mV in DMEM (Table III); the values were slightly lower in DMEM due to the salting effect of the buffer solution and the shielding effect of the serum components.[29] Negatively charged SF particles facilitate the interaction of nanoparticles with positively-charged cells and hence increase the rate and extent of internalization that would be effective for drug delivery.

The FTIR spectra of pure [bmim$^+$][Cl$^-$], SFNs produced from a SIL solution in [bmim$^+$][Cl$^-$] and SFNs obtained using the classical Zhang's method[22] are given in Figure 4. The secondary structure of the SF was predominantly β-sheet in both kinds of particles, with strong peaks at 1626 cm^{-1} (Amide I, C=O Stretching), 1516 cm^{-1} (Amide II, N-H Bending), and 1230 cm^{-1} (Amide III, C-H Stretching), which are characteristic of the β-sheet structure of stable and insoluble SILK II.[27] The peak profile was similar to that of SFNs obtained by precipitation in methanol of SF dissolved in the Ajisawa solvent system.[22,27] Characteristic signals of ILs (1572, 1465, and 1170 cm^{-1}) were absent in the recorded spectrum of SFNs obtained from [bmim$^+$][Cl$^-$], indicating that the IL was efficiently washed out from the particles.

Cell Viability/Cytotoxicity Studies

To study the potential cytotoxicity of the SFNs resulting from different methodologies, MTT assays were performed at two different concentrations (100 and 200 µg/mL), based on similar experiments developed by Kundu *et al.*[28].

The MTT assay is commonly used to evaluate the toxicity of biomaterials, based on their mitochondrial activity, which influences metabolic activity and cell viability[30,31]. To determine the toxicity of the newly synthesized SFNs, we conducted this well-known assay with L929 fibroblast cells, as previously explained. Cell viability was monitored by absorbance measurements obtained in MTT assays. The absorbance values of negative controls (without nanoparticles) were assumed as 100% viability after 24 hours of incubation (Figure 5). No significant differences were found between the cell viability of negative controls and that of the cells incubated with SFN at 100 µg/mL (Tukey, $P > 0.05$), which means that no cytotoxicity was detected at this concentration. However, at 200 µg/mL, the cell viability was decreased significantly in all the treatments when compared with both the negative control (Tukey, $P < 0.05$) and the incubation at 100 µg/mL, for each kind of fibroin nanoparticle (ANOVA, $P < 0.05$). The viability of the cells incubated with particles at 200 µg/mL ranged from 50% to 74%, as shown in Figure 5, while significantly lower viability was detected in the cells exposed to the nanoparticles produced using [emim$^+$][Cl$^-$], compared with the other treatments (Tukey, $P < 0.05$).

Our results agree with the viability data mentioned by Kundu *et al.*[28], who used nanoparticles produced by Zhang's method[22], thus confirming the potential of our new protocol to produce SFNs for biomedical applications.

CONCLUSIONS

Our new methodology involves the application of high-power ultrasounds to a mixture of IL and SF, to enhance the dissolution of the protein. Then, SFNs can be easily obtained from the mixture using a simple desolvation method with polar organic solvents. The regenerated SF micro- or nanoparticles obtained show a degree of β-sheet similar to that of the SF threads and are almost identical to the silk nanoparticles previously described in the literature. After desolvation, the ILs can be efficiently recovered under reduced pressure and reused without losing their solvent property, which considerably enhances the overall efficiency of the process. Large amounts of silk can be turned into biomaterials directly from the dissolved SIL solution, for use in a wide range of applications.

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REFERENCES

1. Omenetto, F. G.; Kaplan, D. L. *Science* **2010**, *329*, 528.
2. Hofmann, S.; Foo, C.; Rossetti, F.; Textor, M.; Vunjak-Novakovic, G.; Kaplan, D. L.; Merkle, H. P.; Meinel, L. *J. Control. Release* **2006**, *111*, 219.
3. Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D. L. *Biomaterials* **2003**, *24*, 401.
4. Asakura, T.; Watanabe, Y.; Uchida, A.; Minagawa, H. *Macromolecules* **1984**, *17*, 1075.
5. Ajisawa, A. *J. Seric. Sci. Jpn.* **1998**, *67*, 91.
6. Zhou, G. Q.; Shao, Z. Z.; Knight, D. P.; Yan, J. P.; Chen, X. *Adv. Mater.* **2009**, *21*, 366.

7. Yan, J. P.; Zhou, G. Q.; Knight, D. P.; Shao, Z. Z.; Chen, X. *Biomacromolecules* **2010**, *11*, 1.
8. Yin, J. W.; Chen, E. Q.; Porter, D.; Shao, Z. Z. *Biomacromolecules* **2010**, *11*, 2890.
9. Rockwood, D. N.; Preda, R. C.; Yücel, T.; Wang, X.; Lovett, M. L.; Kaplan, D. L. *Nat. Protoc.* **2011**, *6*, 1612.
10. Holland, C. A.; Terry, E.; Porter, D.; Vollrath, F. *Polymer* **2007**, *48*, 3388.
11. Aznar-Cervantes, S. D.; Vicente-Cervantes, D.; Meseguer-Olmo, L.; Cenis, J. L.; Lozano-Pérez, A. A. *Mater. Sci. Eng. C* **2013**, *33*, 1945.
12. Phillips, D. M.; Drummy, L. F.; Conrady, D. G.; Fox, D. M.; Naik, R. R.; Stone, M. O.; Trulove, P. C.; De Long, H. C.; Mantz, R. A. *J. Am. Chem. Soc.* **2004**, *126*, 14350.
13. Phillips, D. M.; Drummy, L. F.; Naik, R. R.; De Long, H. C.; Fox, D. M.; Trulove, P. C.; Mantz, R. A. *J. Mater. Chem.* **2005**, *15*, 4206.
14. Gupta, M. K.; Khokhar, S. K.; Phillips, D. M.; Sowards, L. A.; Drummy, L. F.; Kadakia, M. P.; Naik, R. R. *Langmuir* **2007**, *23*, 1315.
15. US, Pat. 7 671 178, **2010**.
16. Goujon, N.; Wang, X.; Rajkowa, R.; Byrne, N. *Chem. Commun.* **2012**, *48*, 1278.
17. Wang, Q.; Yang, Y.; Chen, X.; Shao, Z. *Biomacromolecules* **2012**, *13*, 1875.
18. Rogers, R. D.; Seddon, K. R. *Science* **2003**, *302*, 792.
19. Swatlowsky, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. *J. Am. Chem. Soc.* **2002**, *124*, 4974.
20. Songmin, S.; Lei, Z.; Jintu, F. *Carbohyd. Polym.* **2011**, *86* (2), 462.
21. Mikkola, J.; Kirilin, A.; Tuuf, J. C.; Pranovich, A.; Holmbom, B.; Kustov, L. M. D.; Murzina, Y.; Salmi, T. *Green Chem.* **2007**, *9*, 1229.
22. Zhang, Y. Q.; Shen, W. D.; Xiang, R. L.; Zhuge, L. J.; Gao, W. J.; Wang, W. B. *J Nanopart. Res.* **2007**, *9*, 885.
23. Laemmli, U. K. *Nature* **1970**, *227*, 680.
24. Huynh, M. L.; Russell, P.; Walsh, B. *Methods Mol Biol.* **2009**, *519*, 507.
25. Yamada, H.; Nakao, H.; Takasu, Y.; Tsubouchi, K. *Mater. Sci. Eng. C* **2001**, *14*, 41.
26. Takasu, Y.; Yamada, H.; Tsubouchi, K. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2715.
27. Cao, Z.; Chen, X.; Yao, J.; Huang, L.; Shao, Z. *Soft Matter* **2007**, *3*, 910.
28. Kundu, J.; Chung, Y. l.; Kim, Y. H.; Tae, G.; Kundu, S. C. *Int. J. Pharm* **2010**, *388*, 242.
29. Subia, B.; Kundu, S. C. *Nanotechnology* **2013**, *24*, 035103.
30. Mosmann, T. *J. Immunol. Methods.* **1993**, *95*, 55.
31. Gupta, A. K.; Gupta, M.; Yarwood, S. J.; Curtis, A. S. *J. Control. Release* **2004**, *95*, 197.