Synthesis and characterization of fiber reinforced polymer scaffolds based on natural fibers and polymer for bone tissue engineering application

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Abstract
A wide range of materials and scaffolding fabrication methods for bone tissue engineering have been explored recently. Fiber reinforced polymers (FRP) system appears to be a suitable system. By the exclusive use of biocompatible or bio-absorbable polymers and fibers, novel generation of scaffolds for applications in tissue engineering can be prepared. Mulberry Silk as highlighted natural fiber with its specific economic, mechanical and biological properties has been used for fabrication FRP scaffolds. In this study FRP scaffolds prepared by a combination of silk fibroin polymer, which is another configuration of silk fibers as a porous matrix and silk fibers as the reinforcement element. FRP scaffolds have been fabricated by the freeze-drying method. Microstructure has been analyzed by scanning electron microscopy and the results show an integrative structure. Mechanical properties have been evaluated by universal testing machine. Compressive mechanical modules as well as strength of FRP scaffolds increased about three times in magnitude in comparison with pure fibroin scaffolds. FRP scaffolds had a compressive module of ~3.6 MPa. Osteoblast viability and attachment on FRP scaffolds were investigated in vitro by MTT assay, which showed no cytotoxic response. Additionally, based on SEM results it is concluded that FRP scaffolds provide a good environment for osteoblast attachment.
Keywords**:** Biomaterials; Silk; Fiber reinforced polymer (FRP) composites; Bone tissue engineering

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INTRODUCTION

Bone is an excellent and inimitable composite material designed by nature. It is specifically composed of two major phases; minerals are inserted as reinforcing components while the collagen serves as an organic matrix (Park *et al*., 2007; Murugan and Ramakrishna, 2005). Some structural bone defects can be improved by bone replacement via an artificial substitute. Bone tissue engineers are following resolutions to some essential challenges in bone tissue replacement such as development of a scaffold which presents appropriate mechanical properties throughout the course of biodegradation (Joshua *et al*., 2009). The anisotropy and the exclusive biomechanical properties of bone point to the bio-composites as multifunctional and multi-phase structures for tissue replacement (Ranganathan *et al*., 2010; Cheung *et al*., 2009). Biocomposite materials are good choices for bone replacement due to the selective combination of material's

characteristics such as mechanical properties (Santis *et al*., 2004). Fibers reinforced polymers bio-composites (FRP) consist of biodegradable polymer as matrix and bio-fibers as reinforcing components. These biodegradable fibers generally have low density, high durability as well as enhanced energy retrieval (Tong *et al*., 2002). However, these fibers have trivial cross-sections and cannot be directly used as engineering structures. Therefore, fibers are embedded in the polymer matrix to form bio-composites. The matrix serves as a binder to bind the fibers together and transfer loads to the fibers which are usually more durable than the matrix material. If the matrix is porous as well, this fiber reinforced cellular matrix is suitable as a scaffold (Bühler *et al*., 2008).

Silk as a natural fibrous polymer has promising biocompatibility and mechanical properties for biomedical applications (Hardy and Scheibel, 2010; Vepari and Kaplan, 2007; Wang *et al*., 2006; Altman *et al*., 2003). Silk has been broadly characterized for biocompatibility and results show that biocompatible silk fibroin rivals other biomaterials such as PLA and collagen (Vunjak-Novakovic and Freshney, 2006). Silk provides impressive mechanical properties in combination with lightweight (1.3 g/cm^3). For instance, silk has a remarkable toughness, which is even superior to Nylon, Kevlar and high-tensile steel (Altman *et al*., 2003) Moreover, slow rate of degradation of silk in vitro (Horan *et al*., 2005) and *in vivo* (Wang *et al*., 2008) makes it useful as biodegradable biomaterial for slow tissue ingrowths such as bone. Several different material morphologies whether aqueous solutions, organic solutions or microfibers can be formed from the silk (Collins *et al*., 2009; Hardy *et al*., 2008; Kim *Et al*., 2007). There are protocols that exist to convert natural silk fibers into pure silk fibroin solutions that can be subsequently reconstituted into macro-porous 3D constructions with β-sheet secondary structure (Hofmann *et al*., 2006; Nazarov *et al*., 2004; Um *et al*., 2001). Utilizing silk fibers in combination with gelatin enhanced mechanical properties considering that scaffolds have proper biodegradation as well (Shubhra *et al*., 2011).

MATERIALS AND METHODS

Materials: LiBr, Na_2CO_3, and methanol were supplied by Merck (Germany). Dialysis tube (cut-off value of 12000 Da) was from Sigma (Germany). Mulberry silkworm cocoons were the generous gift from Iranian Silkworm Research Center (ISRC). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) obtained from Gib Co. and Penicillin-streptomycin (Pen-Strep), MTT substrate [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrasodium bromide] from Sigma Chemical Co. Osteoblast cell line (G 292) was purchased from National Cell Bank of Iran, Pasteur Institute.

Fabrication of FP and FR scaffolds: Cocoons of the mulberry silkworm were boiled for 1 hr in a 0.02 M aqueous sodium carbonate solution and then rinsed carefully with cold and hot water to remove the sericin proteins. The degummed silk was dissolved in 9.3 M LiBr at 55°C for 4 h. Fibroin solution was passed through 100 µm mesh to eliminate probable dregs. Then the solution was dialyzed against ultrapure water for 36 h to yield the aqueous fibroin solution. The solution was then lyophilized for 3 days to get the dried storable fibroin. Dried fibroin were resolved to make 2, 4 and 8% w/v solutions for preparation of fibroin pure scaffolds (FP). Solutions were put either into the Teflon molds or 24-well polystyrene plates and then frozen at -24°C for 5 hr and then at -80°C for 2 h. Freezing solutions were then freeze-dried for 12 h leaving a porous matrix. Porous matrixes were immersed in methanol 99.9% for about 1 h to induce crystallization and transforming to β-sheet structure and insolubility in water. FP scaffolds were cautiously rinsed with water to eliminate methanol residue. Scaffolds dried through lyophilizing for more 10 hr. To make fiber reinforced scaffolds (FR), natural continuous silkworm degummed silk fibers were used. Fibers were mixed with 4% w/v solution in different ratios as it describes in Table 1. Fibroin solution with fibers were transferred into proper molds then frozen, lyophilized and treated with methanol in procedure similar to FP samples.

Scanning electron microscopy (SEM): Samples were sputter-coated with gold or carbon. Silk fibers, the pore distributions, sizes and morphologies of scaffolds as well as cell adhesion were observed by Philips scanning electron microscopy, using a XL 30/ESEM field emission gun with an operating voltage of 15 kV.

Compressive mechanical properties and porosity analysis: Porosity is evaluated by liquid displacement method (Gelinsky *et al*., 2008). Hexane which is a non-solvent agent for silk was used as the displacement liquid. Porosity of the scaffolds (ε) was obtained

Table1. Samples' labels and compositions.

Label	FP2	FP4	FP8
Composition	2wt% fibroin	4wt% fibroin	8wt% fibroin
Label	FP30	FP50	FP90
Composition	Fibroin/Fiber 70/30	Fibroin/Fiber 50/50	Fibroin/Fiber 10/90

wt% = g/100 ml

by following the equation of ε (%) = ×100, where V_1 is known volume of hexane, V_2 is total volume of hexane impregnated scaffold after 5 min and V_3 is the residual hexane volume in the cylinder after removal of hexane saturated scaffold. All the mechanical and porosity analysis experiments were performed at least in 6th replicates.

In vitro cell culture: Human osteoblast cell line G 292F was expanded in DMEM low glucose containing 10 U/ml penicillin and 100 µg/ml streptomycin, 9.5% FBS at 37°C in humidified, 5% CO_2/95 % air incubator. For examination of viability of the cells on scaffolds, 5×10^5 cells were seeded onto each disc shaped scaffolds. Briefly, scaffolds were sterilized by UV radiation. Then, the scaffolds were pre-incubated in culture medium for 2 h before seeding. Three scaffolds of each type were seeded with high-concentration cell suspension and incubated for 2 h. Then the volume of the medium for each sample of scaffolds was increased to 1 ml to cover entirely the surface of scaffold. One polystyrene control sample was allocated for each sample, with the same cell number. Culture medium was changed every 2 or 3 days.

MTT assay: Cell viability was quantified by using MTT assay after one week of the culture of the osteoblasts. MTT substrate [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrasodium bromide] is reduced by living cells to a dark-blue formazan by mitochondria. To avoid the miscalculation due to absorption of formazan dye by micro-porous structure of scaffolds, control sample scaffolds were used for normalization. Control sample scaffolds were soaked in the culture medium for 1 h before running the test. The growth medium of each test as well as control samples were replaced with 0.02 ml of MTT (5 mg/ml) solution. Then, 0.1 ml serum-free medium was added to each well for covering the whole surface of the scaffold. The plates were incubated for 4 hr in a humidified atmosphere of 5% CO_2 in the air. Then, the MTT solution was removed and 0.1 ml of dimethyl sulphoxide (DMSO) was added into each well in order to dissolve the formazan crystals in darkness. At this stage, the stain from one sample of each type was transferred to the control wells (those scaffolds which were soaked in the culture medium). The plate was agitated for 20 min in the dark and the viable cells in the colored solution were quantified using a scanning multi-well spectrophotometer at 540 nm.

Attachment and morphology of the cells on scaffolds: Cell-seeded scaffolds were fixed by 3.7% formaldehyde in PBS. Samples were dehydrated using a gradation series of ethanol/distilled water solutions. Critical point drying was performed with a CPD 030 apparatus (BAL-TEC, Liechtenstein, Germany). Finally, samples were prepared for SEM imaging by coating with gold sputtering machine.

Statistical analysis: All experiments were performed in

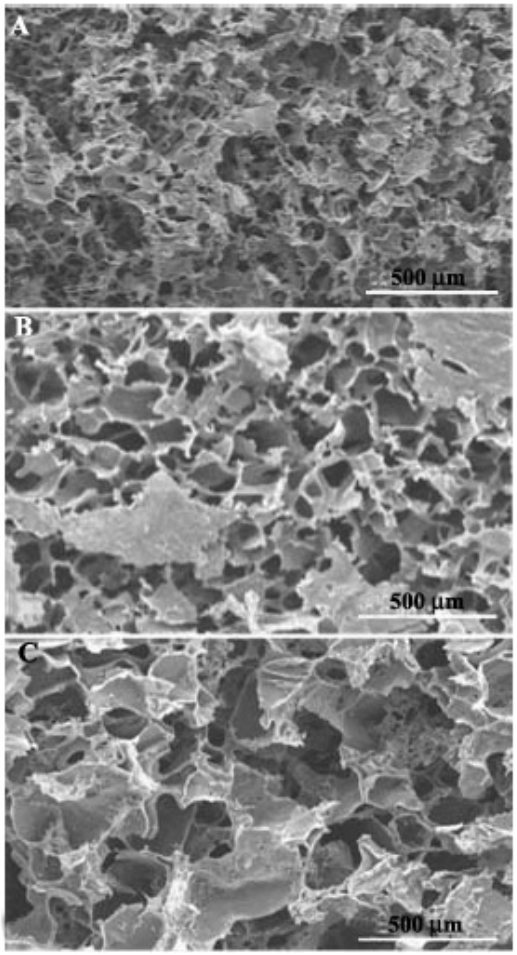

Figure 1. SEM images of A: FP2, B: FP4 and C: FP8. Pore size diminishez by increasing the fibroin concentration.

sixth replicate. The results are presented as means ± Standard deviation (SD). Statistical analysis was performed by SPSS17 using T test. Differences between groups of study with p value = 0.05 were considered significant.

RESULTS

Morphology of fibers and scaffolds: SEM analysis of the FP scaffolds is shown in Figure 1. Freeze-dried pure fibroin solutions in different concentration make a cellular structure which the pore size diminishes by increasing the fibroin concentration. The pore sizes of FP2 scaffolds were approximately 200 to 300 µm while the size of FP8 decreased to 80-100 µm (Fig. 1A-C). Figure 2A and 2B shows continuous degummed silk fibers, which are used as reinforcement in FR scaffolds. The average diameters of the fibers are about 18 µm. Figure 2C and 2D are the SEM images of FR scaffolds. In Figure 2D, the cross section of FR scaffold clearly shows how fibers are embedded in fibroin pores of the matrix.

Mechanical properties and porosity of scaffolds: Porosity and compressive module of both fibroin and fiber reinforced composite samples, which were obtained from strain-stress curves are illustrated in Table 2. Porosity of FP2, FP4 and FP8 scaffolds was estimated 92.3, 87.4 and 83.4% respectively. In fact, there is a contrary relationship between the fibroin concentration and the percentage of porosity. FR30 with fewer fibers has 88.1% porosity while FR50 has 86.8% porosity. However, among FR samples, FR90 with most fibers has the highest porosity of 88.6%. So there is not actual predictable relationship between fiber amounts and porosity. Nevertheless, compressive strength and module directly related to the fiber volume. Compressive module rises up from 0.79 MPa in FR30 to 2.75 in FR50. The same trend is also revealed for compressive strength. Compressive module of FR90 with most fibers is 3.7 MPa and its compressive strength is 1.9 MPa which are significantly higher than samples in FP group with same porosity. In FP samples, both mechanical strength and module decrease by reducing fibroin concentration. This trend is rational due to the amount of ice crystals in fibroin solution composite, which is related to the concentration of solution. The solution, enriched by ice in lower con-

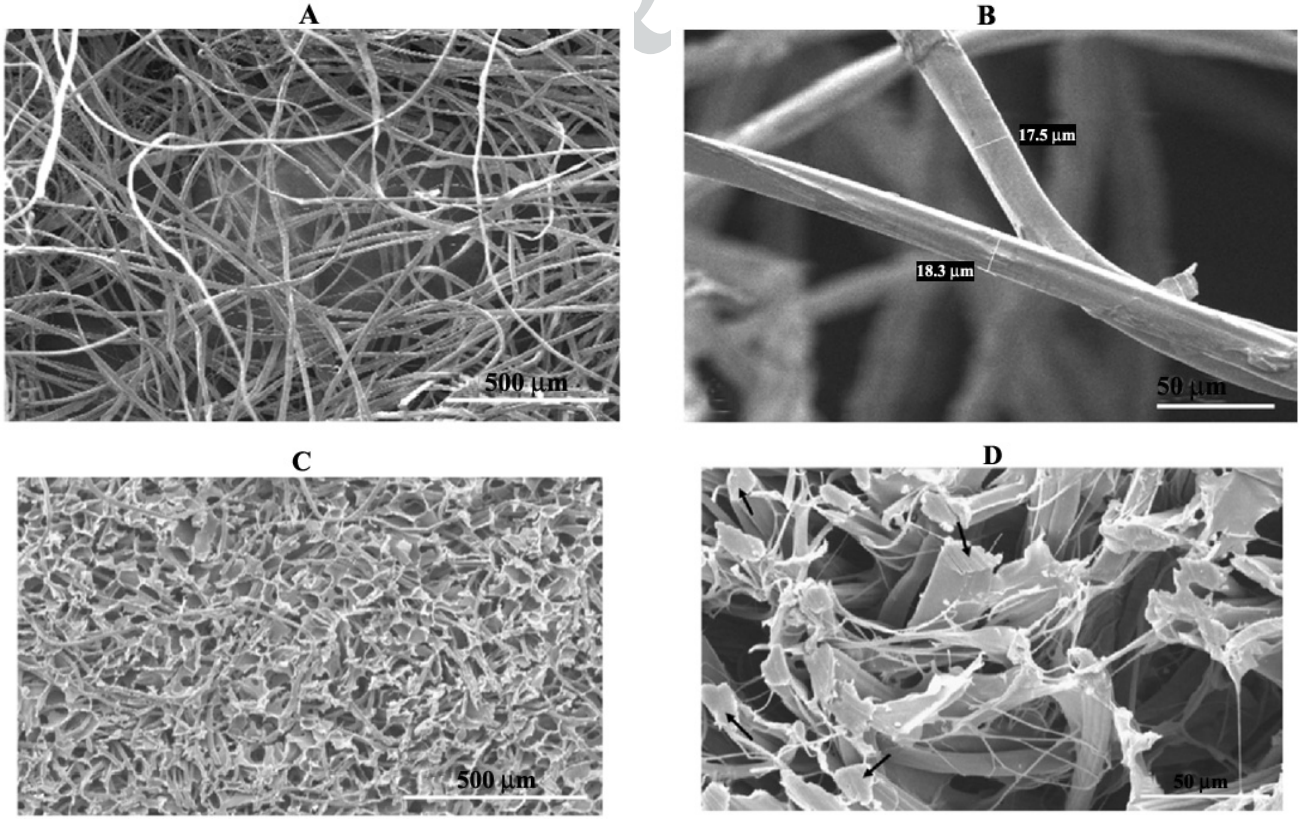

Figure 2. SEM images of A, B: Mulberry silkworm silk fibers 2, C: FR50 surface, D: FR50 cross-section.

Table 2. Mechanical properties and porosity of the scaffolds.

Sample	Porosity (%)	Compressive module (MPa)	Compressive strength (MPa)
FP2	92.3% ± 1.41	0.216 ± 0.011	0.189 ± 0.009
FP4	87.4% ± 1.52	1.114 ± 0.075	1.027± 0.126
FP8	83.4% ± 4.41	2.034 ± 0.749	1.599 ± 0.511
FR30	88.1% ± 0.38	0.795 ± 0.039	0.753 ± 0.0757
FR50	86.8% ± 1.02	2.75 ± 0.482	1.854 ± 0.143
FR90	88.6% ± 0.67	3.69 ± 0.127	1.903 ± 0.044

centration of fibroin (FP2) leaves much more pores. Additionally, much bigger pores can be obtained in FP2 samples in comparison to FP8 samples, which make a less strong structure.

Viability and attachment of Osteoblast on scaffolds: Cellular viability and mitochondrial activity of viable cells on fibroin scaffolds as well as FR scaffolds were determined by MTT assay. Absorbance intensity of formazan indirectly reflects viable cells. Test and control samples were in the same condition excluding the stain in control samples were absorbed by scaffolds just before measurement to eliminate miscalculation due to stain sorption by a scaffold spongy structure. In Figure 3A the significant difference between the cell on polystyrene and control samples (‡ $p \leq 0.05$) shows that, the absorption which is measured for scaffolds could not determine the real formazan amount. Comparing each test sample (scaffolds) with control samples reveals that, although scaffolds has a poorer formazan amount, there is no remarkable difference between them as the p values are all above 0.05. FP2 and FR30 have not as much of difference in comparison with their control samples. Figure 3B shows SEM images of the cell on the both types of scaffolds. Cells have been overextended FP scaffolds. Cells were also stretched over the fibers and attached absolutely on both surface and fiber in reinforced scaffolds.

DISCUSSION

In the present study, we have explored effects of silk fibers as reinforcement on mechanical and biological properties of fibroin scaffolds in the FRP porous system. We found that silk fibers significantly improved

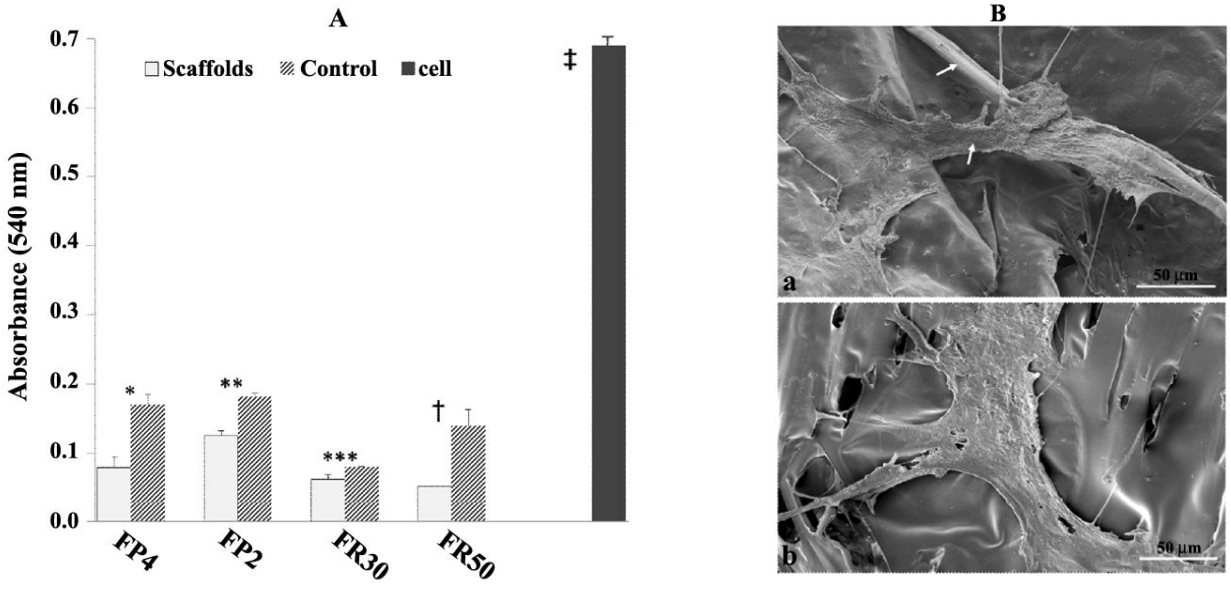

Figure 3. A: Viability of cells seeded on scaffolds and controls after one week. B: a) Osteoblast on FR scaffold, b) Osteoblast on FP scaffold. +A: scaffolds and their controls shows almost the same value, which means of high cell viability.

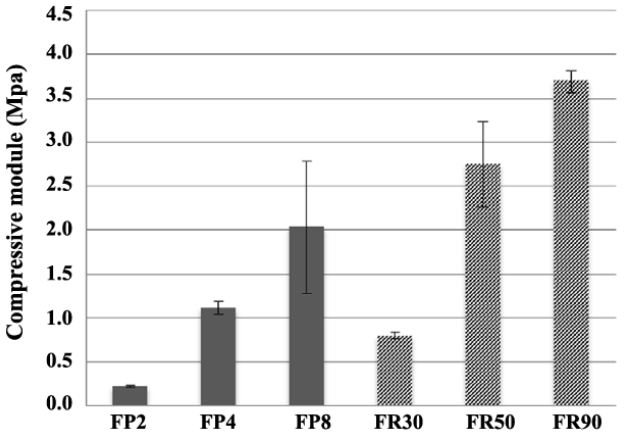

Figure 4. Compressive module of FP and FR samples. FR silk scaffolds also show the greater mechanical properties in comparison with the other traditional scaffolds.

the cellular structure of fibroin with no negative effect on cytocompatibility of the scaffolds. One of the problems with 3D pores scaffolds is the mechanical enhancement. An increase in the pores' volume leads to a reduction in mechanical strength of the scaffold (Rezwan *et al*., 2006; Karageorgiou and Kaplan, 2005). This fiber modification has not much effect on porosity (88.6 % porosity in FR90 samples), while enhanced the compressive module greatly. Figure 4 shows the compressive module of FR and FP samples. The compressive module of FR samples in comparison with the FP scaffolds enhanced largely. The compressive module of FRP scaffolds are close to the cancellous bone which is reported in the range of 2-12 MPa (Hutmacher *et al*., 2007). FR silk scaffolds also show the greater mechanical properties in comparison with the other traditional scaffolds (Rezwan *et al*., 2006).

Our data show that degummed silk which is completely fibroin natural fibers has no cytotoxicity. Cells also find the fibers a suitable surface to attach. This can be found through the SEM image of the cell which was flattened over the fibers. Moreover, we introduced new calculation for traditional MTT assay for those scaffolds which absorb the stain due to their porous structure and capillary systems. There are some solutions which are suggested by other groups for this problem. Some try to detach the cells by enzymes and run the assay over the cells (Huang *et al*., 2006). This method is beneficial to omit the effect of scaffolds however, it is not very convenient and also the enzyme effect is neglected. The other resolution just recommends the contrast qualities between the test and control samples (Gelinsky *et al*., 2008).

CONCLUSIONS

Silk fiber reinforced composite scaffolds were prepared by freeze-drying method and uniform microstructure was achieved. Utilizing natural silk fibers in a porous fibroin system improved mechanical properties of scaffolds while fibers had no negative effect on cells compatibility and viability. Osteoblasts attached on both matrix and fibers in FRP scaffolds. We conclude that appropriate grouping of mechanical properties in a range of spongy bones and biocompatibility of FRP make silk fiber reinforcement scaffolds proper choice for potential uses in bone tissue engineering.

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