ARTICLE

Spider Silk Calcite Composite

Nishaki Mehta and Shantesh Hede

National Toxicology Centre, Pune, India
Correspondence: nishaki_m@hotmail.com & hedeshantesh@hotmail.com

Biomineralisation is a process wherein biomolecules form templates to synthesize advanced materials; for instance in bone and teeth formation. Understanding the superior mechanical properties of flexibility and mechanical strength in naturally occurring spider silk, we propose a simple and economical method of developing smart organic-inorganic composites by growing calcite crystals onto spider silk as a template. This serves to combine the mechanical properties of spider silk with the bio-mineral properties of calcium carbonate (calcite polymorph). This unique composite simulates the strength provided by a bone and the flexibility afforded by a ligament. The composite holds great promise in revolutionizing the sector of artificial joints and prosthesis. Efforts to accomplish these goals through molecular scale mimicry are described in this article.

1) The Template - Spider Silk

Introduction to Spider Silk: Spider silk, a natural polymer made up of repeating amino acid motifs, has fascinated materials scientists for a long time primarily because of its tensile strength, stiffness, extensibility and resistance to breakage (1). Spider silks are composed of fibroin proteins (M_r ~ 2,00,000-3,00,000) that are a combination of the proteins Spidroin 1 and Spidroin 2 (2).

Chemical Composition: Fibroin consists of approximately 42% glycine and 25% alanine as the major amino acids; Spidroin 1 and Spidroin 2 differ mainly in their content of proline and tyrosine residues (3). Gosline *et al.* have made a detailed quantitative study of the mechanical properties of spider silk from *Araneus diadematus* and *Nephila clavipes* and have also given a comparative account of spider silk versus other natural and man made polymers (4). Spider silk is only 35% resilient, but it is nearly as strong as high-tensile steel and requires three times as much energy to break a single thread of spider silk compared to a strand of Kevlar (5). However, unlike man-made materials, spider silk is also known to be extremely elastic and stretches by as much as 500–1,000% (6).

Spiders make silk of various types, numbers, properties and purposes that vary with the species, environment, dietary composition and metabolic status of the organism (7). Not surprisingly, early research has focused primarily on the mechanical properties of spider silk with applications in light and strong composite materials in mind.

Advantage of a Spider Silk based Template: As compared to artificially synthesized polymers, spider silk is simple and economical in terms of harvesting of silk and production of composite, retaining its equally superior mechanical properties (8).

2) Calcium Carbonate (Calcite)

Calcium carbonate ($CaCO_3$) is extensively found in the human body. $CaCO_3$ has three known polymorphs: calcite, aragonite and vaterite. Calcite and aragonite are often found in biominerals, whereas the metastable polymorph vaterite is not generally seen in biological systems. Calcite is thermodynamically the most stable polymorph (9,10). $CaCO_3$ has received considerable attention and biomineralisation principles have been used in the development of a number of biomimetic templates such as Langmuir monolayers, dynamic liquid–liquid interfaces, self-assembled monolayers, lipid bilayer stacks, vesicles and functionalized micro-patterned surfaces for its synthesis

(11–20). Morphology variation and polymorph selectivity of $CaCO_3$ crystals have also been achieved by growth in solution in the presence of suitably designed additives such as proteins extracted from $CaCO_3$-rich organisms or synthetic molecules such as polymers (21).

Experimental Details

Preparation of Spider Silk: The spider *Pholcus phalangoides* (*Pholcidae* family) was kept in a dust-free ventilated box and well fed with house flies for 15 days. The silk was harvested using sharp pointed forceps and then cleaned with isopropyl alcohol.

Preparation of Composite (Figure 1): The silk was kept on a glass substrate, drop coated with 10 mM of Calcium Chloride solution and placed in a dessicator beside a Petridish containing powdered ammonium carbonate (to release carbon dioxide). The liberated carbon dioxide combined with calcium ions attached to the carboxylic acid groups of the silk protein template to form crystals of uniquely oriented calcium carbonate (calcite). This process was a simple method of mineralisation.

During the process of synthesis of the crystals on the silk fiber, it is possible to make it a porous composite. This method would entail altering the calcium carbonate crystal structures, the quantity and the packing orientation of the calcium carbonate layers by changing the conditions during crystal formation (like pH, temperature, calcium ion concentration), alteration of the silk fiber surface morphology and the rate of bubbling of carbon dioxide.

Preparation of sample for analysis: The silk sample, before and after mineralisation, was coated onto a glass substrate for X-ray Diffraction (XRD) measurements and a Silicon Si (111) substrate for Fourier Transformed Infra-Red Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) with Energy Dispersive Analysis of X-rays (EDAX).

Results

<u>1) The rate of calcium carbonate growth onto spider silk.</u>

The data is as follows:

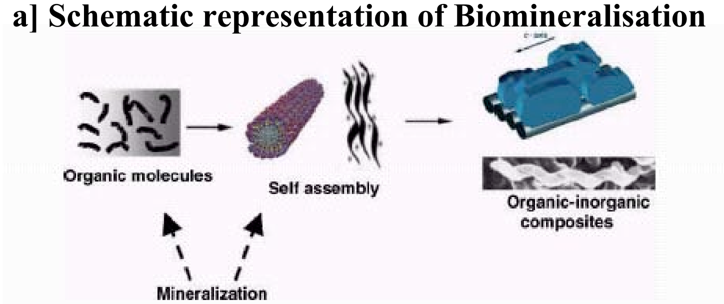
- Weight of the Spider Silk (without mineralisation) = 1.2 mg
- Weight of the silk composite on the 1st day of mineralisation = 1.7mg (41% increase)
- Weight of the silk composite on the 2nd day of mineralisation = 2.6mg (52% increase)
- Weight of the silk composite on the 3rd day of mineralisation = 3.7 mg (42 % increase)

When early stages were observed, there was a sharp rise in calcium carbonate deposition, as high surface area microstructures were formed initially, which promoted additional deposition and patterning of more calcium carbonate crystals.

<u>2) Microscopy-SEM images of spider silk.</u>

Examined before mineralisation, a smooth uncoated strand of spider silk fiber is observed at 10,000X magnification (Figure 2). SEM images of spider silk coated with calcite; the erstwhile smooth strand now shows deposits of the growing calcite crystals (Figure 3). Figures 4 and 5 detail magnification of the composite at 20,000X.

a] Schematic representation of Biomineralisation



b] Schematic representation of the Biomineralisation Procedure

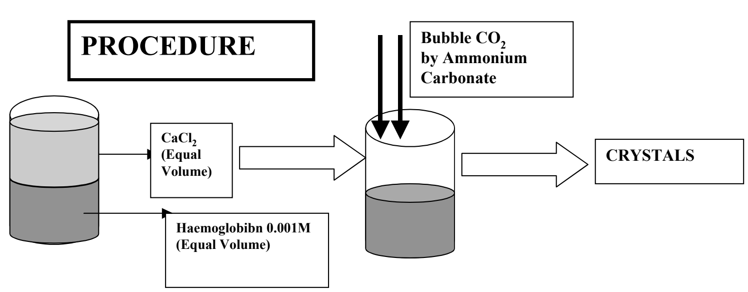


Figure 1. Schematic representation of biomineralisation.

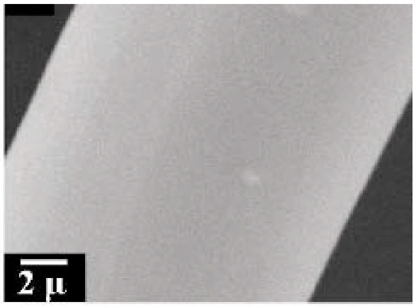


Figure 2 (above). SEM image of spider silk before mineralisation.

Figure 3 (above). SEM image of spider silk after mineralisation.

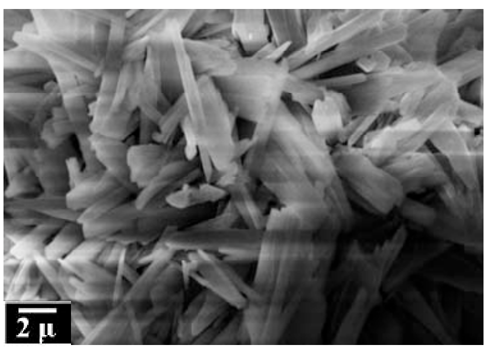


Figure 4 (above). High-magnification SEM image of spider silk mineralisation.

Figure 5 (above). High-magnification SEM image of spider silk mineralisation.

<u>3) FTIR Analysis</u>

The FTIR Spectra Before Mineralisation

The Fourier Transformed Infra-Red Spectroscopy studies were conducted. These studies show the status of different functional groups in a molecule, based on its vibrational conditions in the infra-red region. The carbonyl stretching at 1721 cm^{-1} of the COO^- functional group of carboxylic acid is present in the amino acids of the spider silk (Figure 6, Spectrum 1). By binding with Ca^{2+} ions, the band previously at 1721 cm^{-1} broadens and shifts to 1657 cm^{-1} (Figure 6, Spectrum 2). This confirms the binding of calcium ions in the solution to the COO^- functional group. As FTIR analysis cannot be conducted for the interior of the fiber, this analysis clearly demonstrates that calcium ions do not bind to carboxylic acid groups of certain specific amino acids, but only those amino acids available on the exterior surface of the silk fiber.

In order to facilitate a faster calcium carbonate deposition rate, we suggest two alternatives:

1. Change the crystallization conditions.
2. Synthetically synthesize spider silk fibroin proteins, crystallize calcium carbonate on them, and spin out a mineralized silk fiber. This procedure would result in a fiber containing a higher quantity of calcium carbonate crystals inside and on the surface of the spider silk fibers.

FTIR Spectra After Mineralisation (Figure 7)

The composite shows the two characteristic peaks of Calcite (22) at the wavenumbers of 712 cm^{-1} and 874 cm^{-1}. This implies that calcium carbonate (calcite polymorph) crystals were formed over the spider silk template.

<u>4) X-Ray Diffraction (Figure 8)</u>

The XRD pattern shows characteristic Bragg reflections of Calcite at 58.7°, which is in the orientation of (1 0 4).

<u>5) Energy Dispersive Analysis (Figure 9)</u>

Energy dispersive analysis of X-rays (EDAX) from the crystals shown in Figure 4 and 5 yielded strong Ca, C, N, and O signals together with weaker Na and S signals. This indicates the formation of $CaCO_3$ and also the presence of spider silk in the fiber-like structures.

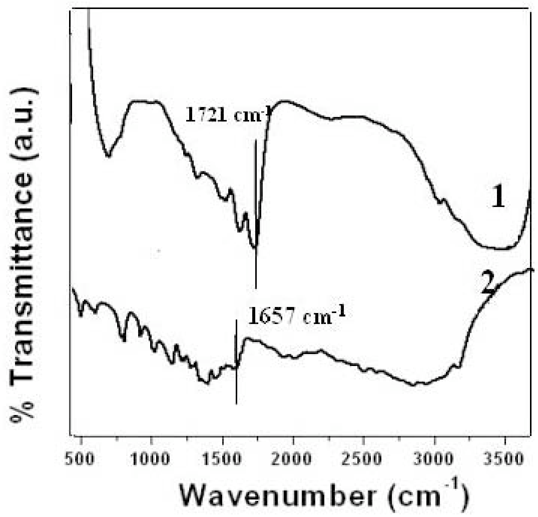


Figure 6. The FTIR spectra of spider silk before mineralization. a.u., arbitrary units.

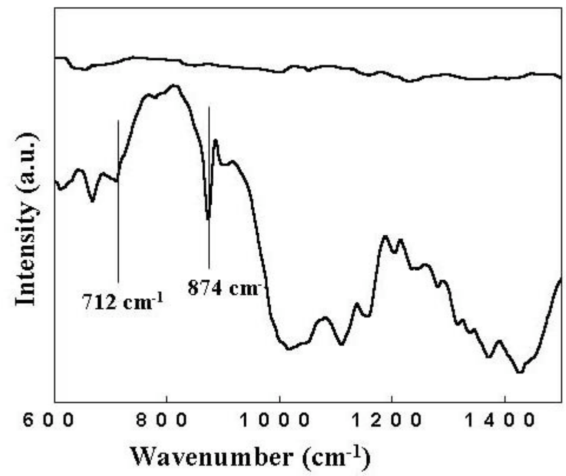


Figure 7. The FTIR spectra of spider silk after mineralisation. a.u., arbitrary units.

Discussion

Falini *et al.* successfully demonstrated the role of chitin and fibroin interactions in bone formation in invertebrates (23). This makes spider silk an appropriate template for development of a smart bone composite. Spider silk proteins have been synthesized using bacteria (24). Based on this discovery, we suggest a further development step, where the synthesis of a specific sequence fibroins can be conducted, to which calcium carbonate or bone like mineral particles can be attached, in order to spin out a mineral-fiber composite.

Fujita *et al.* successfully demonstrated adequate bone bonding and biocompatibility of calcite crystals to rabitt tibiae (25). Calcite is a biodegradable material that bonds to bone without a surface apatite layer. The mechanical bonding provided by the anchoring effect of the newly formed bone into the surface roughness of calcite is considered to be a major factor in calcite-bone bonding. Hence we are inclined to believe that this composite would be a major leap in bridging bone defects in humans along with being widely used in the manufacture of composites.

Conclusion

The spider silk calcite composite developed heralds an avenue to pursue newer appliances in the emerging field of bone implants and

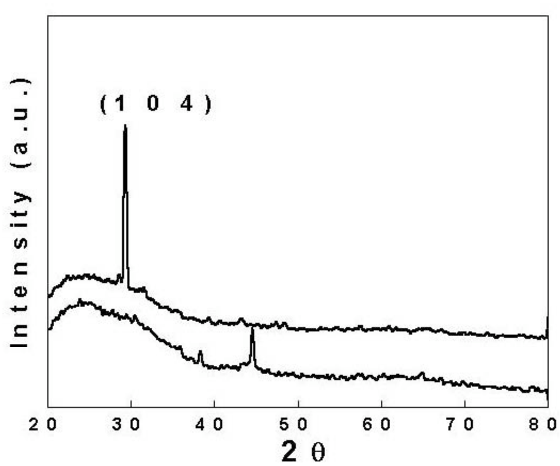


Figure 8. X-ray diffraction of spider silk mineralization. a.u., arbitrary units.

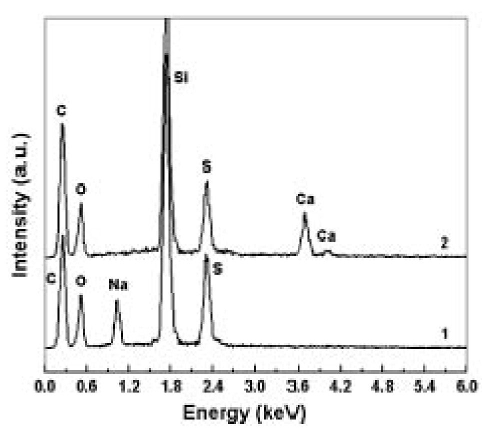


Figure 9. Energy dispersive analysis of X-rays (EDAX) from calcite crystals. a.u., arbitrary units, keV, kiloelectron Volt.

artificial prosthesis. The features of a good bone implant are biocompatibility, bone bonding, strength and cosmesis; and those of prosthesis are strength for weight bearing and flexibility for movement. Calcium carbonate will provide the bony substrate on the spider silk framework and will augur well to improve the alternatives offered to a patient who has undergone amputation or has bone defects that are to be bridged. Further trials are warranted to investigate the same.

References

1. D. A. Tirrell, *Science* **271**, 37 (1996).
2. J. D. van Beek, S. Hess, F. Vollrath, B. H. Meier, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 10266 (2002).
3. M. L. Casem, D. Turner, K. Houchin, *Int. J. Biol. Macromol.* **24**, 103 (1999).
4. J. M. Gosline, P. A. Guerette, C. S. Ortlepp, K. N. Savage, *J. Exp. Biol.* **202**, 3295 (1999).
5. J. C. van Hest, D. A. Tirrell, *Chem. Commun.* 1897 (2001).
6. F. Vollrath, D. T. Edmonds, *Nature* **340**, 305 (1989).
7. R. W. Work, C. T. Young, *J. Arachonol.* **15**, 65 (1987).
8. E.L. Mayes, F. Vollrath, S. Mann*, Adv. Mater.* **10**, 801 (1998).
9. R. Lakshminarayanan, R. M. Kini, S. Valiyaveettil, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 5155 (2002).
10. T. Kato, A. Sugawara, N. Hosoda, *Adv. Mater.* **14**, 869 (2002).
11. B. R. Heywood, S. Mann, *Adv. Mater.* **6**, 9 (1994).
12. S. Rajam *et al., J. Chem. Soc., Faraday Trans.* **87**, 727 (1991).
13. B. R. Heywood, S. Rajam, S. Mann, *J. Chem. Soc., Faraday Trans.* **87**, 735 (1991).
14. S. Mann, B. R. Heywood, S. Rajam, J. D. Birchall, *Nature* **334**, 692 (1988).
15. E. Loste, E. D. Marti, A. Zarbakhsh, F. C. Meldrum, *Langmuir* **19**, 2830 (2003).
16. D. Rautaray *et al.*, *Adv. Mater.* **15**, 1273 (2003).
17. J. Kuther *et al.*, *Chem. Eur. J.* **4**, 1834 (1998).
18. C. Damle, A. Kumar, M. Bhagwat, S. R. Sainkar, M. Sastry, *Langmuir* **18**, 6075 (2002).
19. D. Walsh, S. Mann, *Nature* **377**, 320 (1995).
20. J. Aizenberg, D. A. Muller, J. L. Grazul, D. R. Hamman, *Science* **299**, 1205 (2003).
21. L. Qi, J. Li, J. Ma, *Adv. Mater.* **14**, 300 (2002).
22. I. Sondi, E. Matijevic, *J. Colloid Interface Sci.* **238**, 208 (2001). ,
23. G. Falini, S. Weiner, L. Addadi *et al.*, *Calcified Tissue International* **72**, 548 (2003).
24. S. R. Fahnestock, Z. Yao, L. A. Bedzyk, *Rev. Mol. Biotechnol.* **74**, 105 (2000).
25. F. Fujita *et al.*, *J. Biomed. Mater. Res.* **25**, 991 (1991).