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Review Article

Will silk fibroin nanofiber scaffolds ever hold a useful place in Translational Regenerative Medicine?

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Abstract: Presently, some view silk fibroin-based biomaterials as obsolete, being outperformed by a host of newly discovered biomaterials. But several lines of evidence support the notion that silk fibroin proteins, especially those from *B. mori* and spiders and their recombinant forms, particularly in the form of electrospun nanofiber scaffolds, still represent promising tools for human tissue engineering/regeneration. Inevitably, the allure of recently reported biomaterials turns away many scientists and resources from the aim of more deeply elucidating the biological interactions of the various kinds of silk fibroin nanofiber scaffolds *in vivo*. But, even the biological features of newly reported biomaterials are not investigated in adequate depth. Hence, collaborative efforts among biomaterialists, biomedical experts, and private firms must be undertaken on a much greater scale than hitherto done to assess the real usefulness of silk fibroin proteins, thereby allowing or denying their useful introduction into the fields of Translational Regenerative Medicine.

Keywords: Silk fibroin proteins, electrospun nanofibres, human tissue engineering / regeneration, biomaterials, translational medicine

The ideal gold standard for purposes of human tissue engineering/regeneration are deemed to be biomaterial nanofiber scaffolds endowed with a set of optimized features in connection with surface properties, biocompatibility, gas permeability, biodegradability, mechanical strength, immunogenicity, foreign body response (FBR), and release of growth factor(s) and/or drug(s). Amid the biomaterials candidating for such nanofiber scaffolds, the silk fibroin proteins (SFs), have been objects of a growing interest [1,2], as demonstrated by the trend of the number of publications appeared in the scientific literature during the last ten years (**Figure 1**). SFs are natural proteins produced by wild and domesticated silkworms, spiders, honeybees, wasps, and ants [3-7]. Variants of SFs have been genetically engineered to produce novel biomaterials [8]. They share repetitive amino acid sequences of the poly(Ala) or poly (Gly-Ala) type arranged in antiparallel beta-sheet structures [9], are biodegradable *in vitro* and *in vivo* [10-13], very little inflammogenic [14,15] and can be electrospun into pure SF nanofibers [16-22] or blended with other biomaterials, e.g. poly(L-lactic acid), poly(ethylene oxide), poly(ε-caprolactone), hydroxybutyl chitosan, heparin, collagen, gelatin, etc. [23-37].

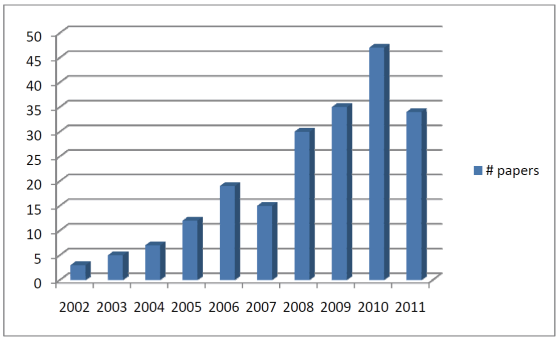

Figure 1. Trend of publications on SF nanofiber scaffolds for biomedical applications

The electrospun SF scaffolds have been reported to exhibit good cell biocompatibility [38] and their structure and physicochemical fea-

tures were shown be deeply influenced by the treatments SF undergoes prior to and/or during electrospinning [4,21,25,39-41]. Application of SF nanofiber scaffolds has been suggested for the engineering and regeneration of both soft tissues, like vascular grafts, nerves, skin wounds [42-54], and hard tissues, like tendons, ligaments, bone and cartilage [16,55-65], although in the latter instances SF was also used as microfiber or sponge and mixed with other biomaterials. In these tissue regeneration attempts bone marrow mesenchymal stem cells have often been seeded onto the scaffolds to observe their differentiation into the several types of the connective tissue or into Schwann peripheral nerve cells [49]. To enhance cell adhesion, spreading, and proliferation, SF nanofiber scaffolds were also functionalized with various bioactive peptides (RGD, BMP, etc.) [16,54,66].

Notably, most if not all of the authors claim to have been successful in their endeavors. So, why the SF nanofiber scaffolds have not as yet found their way to the application in the clinical settings?

What is missing?

It should not be overlooked that most of the published studies on the topic mainly concern the still ongoing efforts to improve the technical procedures used to prepare the scaffolds and hence the scaffolds themselves and to assess their physicochemical properties. Conversely, the interactions of the scaffolds with living cells *in vitro* or *in vivo*, especially the long-term ones, often if not mostly appear as shallow appendages of the biomaterialistic studies. Is this an indication that we are still in too an early phase to have set up appropriate scaffolds? This scientific "immaturity" is moreover compounded by the fact that the biological tests have been preferentially carried out on rodent cells of rats or mice, indeed imperfect mirrors of the corresponding human tissues. In such models, the local and general responses to the implanted SF nanofiber scaffolds, as well as the timing and rates of the degradation of the latter have been poorly assessed. On the whole, while the technically complex questions related to the characteristics and production of the nanofiber scaffolds keep being targeted, the as well or even more intricate biological interactions of the same scaffolds with living cells, tissues, and organisms have by and large remained in the shades. This has somewhat delayed or hampered the onset of an applicative era for the SF nanofiber scaffolds. As a consequence, in some people, this has generated the idea that SFs are old-fashioned biomaterial relics, interesting maybe but not so functional: hence, they should be superseded by novel, more fashionable, and more readily applicable biomaterials.

What the remedy might be?

In the opinion of the authors, this negative outlook is hasty and not founded enough. First of all, it should be recalled here that many human proteins expressed by both epithelial and connective tissue cells exhibit biologically significant (*i.e.*, with very low E-Values and *P* values) homology sequences with heavy-chain *B. mori* SFs (just as an example, see the 49 human proteins listed in **Table 1**). Such proteomic data underlie the reason why surgical stitches made of degummed (sericin-deprived) SF are not immunogenic in humans [67]. In addition, SF-based scaffolds have been shown to favor angiogenesis, an feature essential for tissue repair/regeneration [15,68]. Hence, these important pieces of evidence are not to be overlooked in the perspective of the clinical application of SF-based scaffolds. Moreover, a huge hoard of promising data concerning SF-based biomaterials has been accumulating during recent years. To make the jump to the applicative settings some more technological improvement leading to more robust and reliable production techniques, a deeper knowledge of structure/function relationship of SF nanofiber scaffolds and, as mentioned above, an increase in the understanding of the biological interactions are all is needed. Therefore, to seriously overcome such hurdles and reach a definitive assessment of the opportunities (or, unluckily, lack of them) of the SF nanofiber scaffolds in human tissue repair/regeneration, it should be realized that biomaterialists and cell/tissue/animal/human biology experts must solidly cooperate with each other while absolutely avoiding the hard to resist temptation to assume each other's role, as the skills required for either job are highly complex and specific. By each one sticking to his/her respective role, it would be easier to carry out compound and articulated research projects aimed at scalarly optimizing and standardizing the SF nanofiber scaffolds according to the targets they are aimed at (wound healing, chondrogenesis, osteogenesis, etc.). These major research projects would entail several successive

Table 1. A partial list of human proteins endowed with biologically significant sequence homologies to heavy-chain SF from *B. mori*

Accession	Entry name	Protein names	Length	Identity	Score	E-Value	Gene names
Q86YZ3	HORN_HUMAN	Hornerin	2850	32.00%	2239	0	HRNR S100A18
Q5DT20	Q5DT20_HUMAN	Hornerin	2850	32.00%	2238	0	HRNR
Q7Z5P9	MUC19_HUMAN	Mucin-19 (MUC-19)	6254	23.00%	1648	0	MUC19
Q7Z5P9-2	MUC19_HUMAN	Isoform 2 of Mucin-19	5936	23.00%	1632	1.00E-180	MUC19
Q5D862	FILA2_HUMAN	Filaggrin-2 (FLG-2) /Ifapsoriasin	2391	25.00%	943	1.00E-100	FLG2 IFPS
P15502-10	ELN_HUMAN	Isoform 10 of Elastin	759	35.00%	672	9.00E-69	ELN
P15502-9	ELN_HUMAN	Isoform 9 of Elastin	792	34.00%	667	3.00E-68	ELN
P15502	ELN_HUMAN	Elastin (Tropoelastin)	786	33.00%	663	1.00E-67	ELN
P15502-4	ELN_HUMAN	Isoform 4 of Elastin	757	32.00%	651	2.00E-66	ELN
A7L3I8	A7L3I8_HUMAN	Elastin	757	32.00%	651	2.00E-66	ELN
P15502-1	ELN_HUMAN	Isoform 1 of Elastin	730	33.00%	647	7.00E-66	ELN
P15502-2	ELN_HUMAN	Isoform 2 of Elastin	724	35.00%	643	2.00E-65	ELN
E7EN65	E7EN65_HUMAN	Uncharacterized protein	714	35.00%	640	4.00E-65	ELN
E7ENM0	E7ENM0_HUMAN	Uncharacterized protein	706	34.00%	635	2.00E-64	ELN
B4E3S4	B4E3S4_HUMAN	cDNA FLJ56005, highly similar to Elastin	706	34.00%	632	4.00E-64	
P15502-13	ELN_HUMAN	Isoform 13 of Elastin	705	34.00%	629	8.00E-64	ELN
P15502-8	ELN_HUMAN	Isoform 8 of Elastin	658	35.00%	627	1.00E-63	ELN
P15502-5	ELN_HUMAN	Isoform 5 of Elastin	711	34.00%	627	1.00E-63	ELN
P15502-6	ELN_HUMAN	Isoform 6 of Elastin	687	35.00%	621	7.00E-63	ELN
P15502-12	ELN_HUMAN	Isoform 12 of Elastin	692	34.00%	607	3.00E-61	ELN
B4DK08	B4DK08_HUMAN	cDNA FLJ59555, highly similar to Trophinin	962	24.00%	605	5.00E-61	
P15502-7	ELN_HUMAN	Isoform 7 of Elastin	677	36.00%	597	4.00E-60	ELN
B3KNM5	B3KNM5_HUMAN	cDNA FLJ14955 fis, highly similar to	814	24.00%	596	6.00E-60	
Q12816	TROP_HUMAN	Trophinin (MAGE-D3 antigen)	1431	24.00%	592	2.00E-59	TRO KIAA1114
F5GY27	F5GY27_HUMAN	Uncharacterized protein	962	24.00%	592	2.00E-59	TRO
B1AKE9	B1AKE9_HUMAN	Trophinin (Uncharacterized protein)	1034	24.00%	592	2.00E-59	TRO RP6-14C6.1-
E9PBM4	E9PBM4_HUMAN	Uncharacterized protein	663	35.00%	580	4.00E-58	ELN
B3KSR2	B3KSR2_HUMAN	cDNA FLJ36819 fis, highly similar to Elastin	663	34.00%	580	4.00E-58	
Q8NBI4	Q8NBI4_HUMAN	cDNA PSEC0254 fis,similar to Elastin	643	35.00%	575	2.00E-57	
P15502-11	ELN_HUMAN	Isoform 11 of Elastin	570	37.00%	573	3.00E-57	ELN
B3KRT8	B3KRT8_HUMAN	Elastin, isoform CRA_I similar to Elastin	617	35.00%	570	6.00E-57	ELN hCG_18037
P20930	FILA_HUMAN	Filaggrin	4061	19.00%	549	2.00E-54	FLG
P02461	CO3A1_HUMAN	Collagen alpha-1(III) chain	1466	24.00%	482	9.00E-47	COL3A1
D2JYH5	D2JYH5_HUMAN	Collagen, type III, alpha 1	1466	24.00%	482	9.00E-47	COL3A1
Q6FHY3	Q6FHY3_HUMAN	LOR protein	316	41.00%	468	4.00E-45	LOR
P23490	LORI_HUMAN	Loricrin	312	40.00%	456	1.00E-43	LOR LRN
P02452	CO1A1_HUMAN	Collagen alpha-1(I) chain	1464	26.00%	436	2.00E-41	COL1A1
P08123	CO1A2_HUMAN	Collagen alpha-2(I) chain	1366	24.00%	432	6.00E-41	COL1A2
F5H299	F5H299_HUMAN	Uncharacterized protein	1367	24.00%	430	1.00E-40	COL1A2
Q8N2G0	Q8N2G0_HUMAN	cDNA PSEC0191 fis, similar to Elastin	472	38.00%	417	3.00E-39	
Q6LAN8	Q6LAN8_HUMAN	Collagen type I alpha 1 (Fragment)	1069	25.00%	406	6.00E-38	COL1A1
P02458-1	CO2A1_HUMAN	Isoform 1 of Collagen alpha-1(II) chain	1418	23.00%	390	4.00E-36	COL2A1
P02458	CO2A1_HUMAN	Collagen alpha-1(II) chain/ Chondrocalcin]	1487	23.00%	390	4.00E-36	COL2A1
Q6ZUN2	Q6ZUN2_HUMAN	cDNA FLJ43523 fis, similar to Elastin	559	33.00%	381	5.00E-35	
P35527	K1C9_HUMAN	Keratin, type I cytoskeletal 9 (CK-9)	623	47.00%	378	1.00E-34	KRT9
B4DS47	B4DS47_HUMAN	cDNA FLJ59745, highly similar to Trophinin	795	22.00%	377	1.00E-34	
O60354	O60354_HUMAN	Loricrin	338	41.00%	356	4.00E-32	
P02461-2	CO3A1_HUMAN	Isoform 2 of Collagen alpha-1(III) chain	1163	23.00%	354	6.00E-32	COL3A1

These data were obtained using the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/cgi-bin/blast/blast.pl). Consult http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html for the meanings of Scores and E-Values. Note that the *P* levels of statistical significance (not shown) and the E-Values very closely match when the latter are <0.01.

rounds of structural and functional improvement of the SF nanofiber scaffolds based upon the results gained by assessing both *in vitro* and *in vivo* the biological properties of their previous versions. Thus, through the proper use of the specific expertise of either group, the time required to attain optimized (or nearly so) scaffolds should be significantly shortened. This recursive testing and optimization of the scaffolds would finally open the way to preclinical testing of the optimized scaffolds in large mammals (e.g. dog, sheep, etc.). The assessment of the local tissue engineering/regeneration and local inflammatory and FBR responses and of

general immunological (if any) reactions to the grafted scaffolds in such mammals would allow their definitive optimization. At this step, phase I clinical trials on selected cohorts of human patients would be feasible and fully justified, and their results would (or would not) lead further to phase II and III clinical trials and, eventually, to the approval of the scaffolds in their final formulations for human use by the FDA. It is implicit that to implement such a scheme large monetary resources would be needed and the collaboration between international research groups more than welcome would be mandatory.

Conclusions

On the basis of the available lines of evidence, SF proteins, especially the most studied ones from *B. mori* and spiders, and their genetically recombinant forms can still be considered as promising tools and not outdated biomaterials, for human tissue engineering/regeneration. But it is clear that efforts on a much greater scale than up till now are needed to definitively assess the effective usefulness of such tools, thereby allowing (or negating) their successful introduction into the fields of Translational Regenerative Medicine (i.e. from the lab bench to the patient's bed). A factor complicating the picture is that the SF nanofiber scaffolds may contain additional biomaterials and be molded into manifold morphologies according to their specific aims. No doubt, these efforts require the wide cooperation of international groups with deep specific expertise in their respective fields, i.e. biomaterial technologies and animal and human cell and tissue biopathology, respectively. To such projects private firms should also partake given the huge economic interests involved if the translation of SF-based nanofiber scaffolds to the clinical settings is made possible.

Unavoidably, the incessant discovery of novel biomaterials other than the SFs, while advancing our knowledge in basic sciences, takes away scientists and resources from more deeply focusing on the potential applications of the SF nanofiber scaffolds. But the biological features of these newly discovered biomaterials are not generally studied in greater depth either. Hence, from the standpoint of Translational Regenerative Medicine, it remains undecided whether eventually they might or not be superior to the SF nanofibers. Therefore, although the currently available choices are manifold, it is conceivable that a considerable loss for Human (and Veterinary Medicine) could stem from not fully investigating the real clinical opportunities well devised and tested SF nanofiber scaffolds would be likely to offer.

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