Exploring North East India's non-mulberry silk based bioinks for three dimensional bioprinting

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Three-dimensional bioprinting has catapulted research in the domain of tissue engineering and regenerative medicine to newer heights in recent years. In this milieu, silk-based bioink has garnered tremendous research thrust. Mulberry silkworm silk fibroin has found profound impetus for formulating biocompatible and mechanically robust bioink with high-cell loading potency, used in the fabrication of 3D bio-constructs for prospective clinical applications. Pertinently, North East India's non-mulberry silk varieties 'muga' and 'eri', endowed with specific cell-binding RGD sequence, exhibit special biomaterial-attributes including better mechanical resilience than their mulberry counterparts. The recent exploitation of the former for the formulation of novel bioinks to fabricate 3D constructs for prospective meniscus, cartilage and osteochondral tissue repair merits special mention. Their self-gelling attribute permits the evasion of the use of conventional toxic chemical cross-linkers. With prospective application in the niche of regenerative medicine, these non-mulberry silk varieties seem to have seeded new anticipations vis-à-vis increasing cases of degenerative diseases and associated morbidity.

Three-dimensional bioprinting, representing a nexus of interdisciplinary expertise, involves the fabrication of 3D structures via layer-by-layer specific positioning of biological materials, biomolecules/biochemicals and living cells, the placement of the functional constituents being spatially controlled[1,2]. Approaches of biomimicry, mini-tissue building blocks and autonomous self-assemblage have been adroitly employed for the fabrication of 3D functional human constructs (e.g. multi-layered skin, tracheal splints, heart tissue, etc.), exhibiting biological and mechanical attributes, befitting the clinical restoration of tissue/organ function[3]. These seem to be highly pertinent in the context of incessantly increasing morbidity due to degenerative diseases. Albeit highly potential, the selection of appropriate materials, cell types, growth and differentiation factors as well as sensitivity of the cells and overall complicacy involved in the fabrication of tissues are some of the practical inconveniencies of the technology[2]. Printability, biocompatibility, appropriate structural and mechanical attributes, degradation kinetics adjustable to the capacity of the cells to generate their extracellular matrix (ECM), nontoxic degradation by-products, and most importantly, potency to mimic the tissue-specific endogenenous material properties are the hallmark signatures of an ideal biomaterial to function as a bioink, the core-material for 3D bioprinting. In recent years, researchers have resorted to silkworm protein for formulating bioink[4,5].

The impetus received by silk in the domain of tissue engineering and allied biotechnological applications needs no further elaboration in the backdrop of its biocompatibility, amenability to processing into multiple formats (scaffolds, nanoparticles, hydrogels, electrospun membranes, etc.), tunable biodegradability and mechanical robustness[6–10]. In the realm of 3D bioprinting, exploration of silk, in particular that extracted from *Bombyx mori* (domesticated, mulberry silk variety), has been a recent research thrust[4,5]. Pertinently, biospinning of the silkworm cocoon, in accordance to the dictates of microfluidics, perhaps could be projected as quintessential evidence of nature's self-endeavours in the domain of 3D bioprinting[11].

Silkworm silk consists of the fibroin (SF) core (used in bioprinting), encased by the glue-like sericin (SS; that is degummed using approaches like alkali-degradation, autoclaving, etc.) prior to formulation of bioink[4,12]. However, low viscosity of the extracted SF solution is a serious practical problem and as such, rheological attributes must be adjusted to ensure printability[1]. Post SF purification through dialysis, the regenerated SF solution is subjected to evaporation or re-dissolution in organic solvents (1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), formic acid, etc.) to augment the viscosity[5]. However, such solvents could usher in catastrophic effect on cell viability and may even lead to further scissoring of the fibroin protein. As an alternative, augmentation of free-standing attributes, plasticity and viscosity of SF-based bioinks could be facilitated through blending with other high-viscosity biomaterials like gelatin, chitosan, alginate, etc. Protein conformational transition (induction of beta-sheet structure) in regenerated SF, mediated by approaches involving biocatalysts, sonication, modulations in temperature and pH values, salting, photo-crosslinking as well as (toxic) cross-linkers, is exploited to adjust the mechanical attributes of silk-based bioinks. Besides critical rheological features, swelling ratio, surface tension as well as cell-encapsulating or growth factors doping potency of silk-based bioinks are pertinent from the perspective of cell viability, adhesion, proliferation, differentiation and maturation[5]. Choosing appropriate cell-seeding technology as well as optimization of degradation rate of the constructs with respect to the speed of neo-tissue formation are critical. Nevertheless, availability of considerably large amount of silk sources (silkworm cocoons in particular), intrinsic hydration properties and remarkable strength and toughness of silk, availability of diverse protocols for cross-linking or sol-to-gel induction, tunable biodegradation, ease of modifying the structure and surface chemistry, and

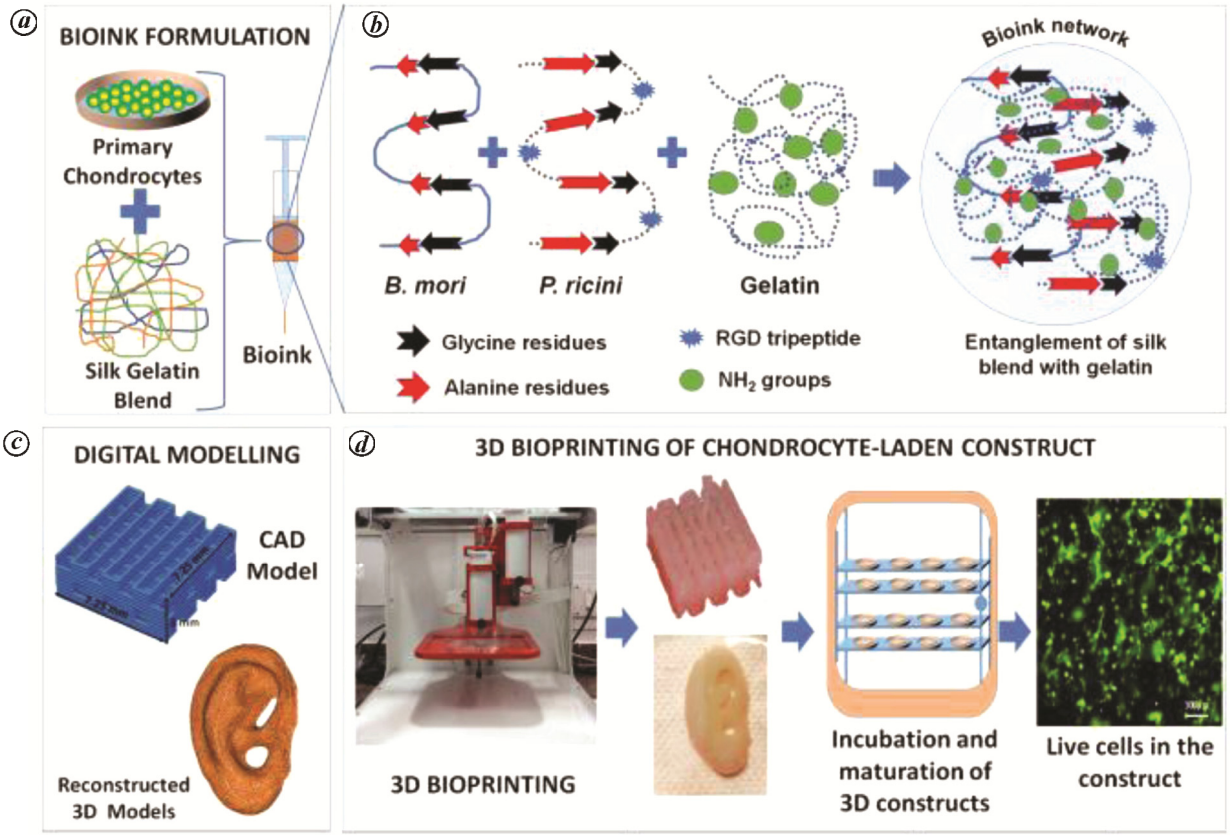

Figure 1. Scheme of the study showing (***a***) bioink formulation, (***b***) entanglement and interaction of biopolymers, (***c***) digital modelling and, (***d***) 3D bioprinting and maturation of cell-laden construct[12] (Reproduced with permission. Copyright © 2019 American Chemical Society.)

conferring of a biomimicking micromilieu to support high cellular viability project silk as an appropriate material for formulating next-gen 3D bioink[1,4,5].

North East India, in particular the Brahmaputra valley of Assam, shelters the endemic non-mulberry silk worm variety *Antheraea assamensis* that produces the golden yellow silk yarn (muga), reverberating the opulence and dynamic culture of the region[6]. On the other hand, spun silk produced by another non-mulberry variety, *Philosamia ricini* (eri) also demands special merit. The presence of intrinsic cell-binding RGD sequences within these non-mulberry SF proteins and greater mechanical robustness in terms of mechanical strength in comparison to *B. mori* silk have endowed distinctive edge to the former in the domain of tissue engineering and regenerative medicine[6–10]. Nutritional attributes as well as environmental aspects dictate the structural and biophysico-chemical features of various silk varieties. The varied compositional abundance of amino acids endows distinct attributes to the mulberry and non-mulberry SF proteins[6,7]. In this context, researchers have resorted to the self-gelling behaviour (without the use of any external stimulus) of the non-mulberry silk proteins extracted from the glands of these worms with the mulberry variety in the presence of gelatin as a bulking agent (that participates in entanglement and physical crosslinking with SF) to fabricate novel bioinks with high print-fidelity[1,12,13].

Recently, Mandal and co-workers have documented a bioink based on *B. mori* SF-*P. ricini* SF–gelatin cocktail recipe for prospective 3D bioprinting of cartilage tissue (Figure 1)[12]. The high viscosity of gelatin and shear thinning feature of silk augmented the printability of the bioink. Apposite microarchitecture, swelling nature and porosity, degradability as well as desirable biomechanical attributes supported a good viability of cells (homogeneously distributed in the bioprinted grids), high production of ECM, upregulated expression of chondrogenic genes and *in vitro* biocompatibility. Post *in vivo* subcutaneous implantation, cell migration was documented into the acellular construct, with minimal immune response, as attested by low infiltration of CD68-positive macrophages. It is worth to mention at this juncture that cartilage tissue lacks innate self-regeneration capability and 3D bioprinted structures, as reported[12] could pave the way for treatment of cartilage tissue degeneration. Another facet of the study was the demonstration of achievability to print anatomical structures (human ear, in the report) via adjusted extrudability at ample resolution.

On a similar vein, addressing patient-specific meniscus injury to counteract complications like knee osteoarthritis using tissue engineering and 3D silk-based bioprinting protocol[1] is quite compelling from the perspective of biomaterials research. Based on similar self-gelling approach as reported in the previously cited study[12], recent endeavours have been directed to explore the prospects of ‘muga’ SF in lieu of ‘eri’ for formulating the bioink. The group succeeded in fabricating a 3D-printed meniscus scaffold

with optimal swelling, degradation and mechanical profiles, supporting the proliferation and phenotype maintenance of seeded fibrochondrocytes with augmented synthesis of glycosaminoglycan and total collagen. The scaffolds, biomimicking the internal and bulk design of the menisci and endowed with amenable *in vitro* and *in vivo* immuno-compatibility may be assessed as a prospective substitute to the current meniscus-repairing approaches.

A recent study has also been directed towards addressing the heterogeneity of phases and anisotropic complexity in osteochondral interface using bioprinted osteochondral construct that supported the spatial maturation and differentiation of encapsulated stem cells towards osteogenic and chondrogenic lineages[13]. The construct was fabricated using (a) *B. mori* and *A. assamensis* SF-based multifunctional cartilage bioink endowed with the desirable facets of shear thinning, fast thixotropic recovery and commendable shape-fidelity for printing, and (b) bone ink with hierarchically biomimetic strontium-doped nano-apatite as the ceramic additive.

As substantiated by the studies cited here, silk-based 3D bioprinting, though in its burgeoning stage, could usher in new hopes in the biomedical domain. Research on the use of non-mulberry-based silk for 3D bioinks has just begun. It is anticipated that a plethora of silk-based 3D printed constructs would be available as 'off-the-shelf' products for clinical applications in the future. Exploitation of such 3D printed silk materials for allied applications like fabricating devices for microfluidics, biophotonic components as well as formulation of ink, capable of detecting microbial contamination and development of fire-retardant gels, based on suitable additives and blending materials is envisaged to open new research portals.

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