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Silk-based microcarriers: current developments and future perspectives

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Abstract: Cell-seeded microcarriers (MCs) are currently one of the most promising topics in biotechnology. These systems are supportive structures for cell growth and expansion that allow efficient nutrient and gas transfer between the media and the attached cells. Silk proteins have been increasingly used for this purpose in the past few years due to their biocompatibility, biodegradability and non-toxicity. To date, several silk fibroin spherical MCs in combination with alginate, gelatin and calcium phosphates have been reported with very interesting outcomes. In addition, other silk-based three-dimensional structures such as microparticles with chitosan and collagen, as well as organoids, have been increasingly studied. In this study, the physicochemical and biological properties of these biomaterials, as well as the recent methodologies for their processing and for cell culture, are discussed. The potential biomedical applications are also addressed. In addition, an analysis of the future perspectives is presented, where the potential of innovative silk-based MCs processing technologies is highlighted.

1 Introduction

1.1 Role of microcarriers (MCs) in modern biotechnology

The global Biomaterials Market is expected to reach USD 139 billion by 2022, at an annual growth rate of over 11.8% [1]. Nowadays, the application of synthetic or natural materials is essential to respond to the increasing cases of chronic diseases, trauma injuries leading consequently to market growth. Advanced biomaterials for cell adhesion, proliferation and differentiation are used to improve the functionality of damaged tissues and organs [2]. Among these, small and stable and spherical MCs have played an important role in the expansion and differentiation of anchorage-dependent cells, enabling potential scale-up of stem cell-derived products in large bioreactors [3, 4]. A wide range of MCs is commercially available and has different physicochemical parameters, such as degree of porosity, chemical composition, surface topography and diameter (comprised between 100 and 400 μm) [5, 6].

MCs allow the investigation of cell behaviour in vitro in a three-dimensional (3D) environment similar to their natural surroundings in vivo and may provide an alternative to animal studies [5, 7, 8]. Moreover, the microtissues formed through MCs and cells can be directly delivered to the site of the defect, thus eliminating the digestion of cells before transfer from a monolayer culture [3, 9].

Although current studies on silk-based MCs focus on microparticles, downsizing some of these systems onto nanocarriers, is an area of interest in biomedical engineering. Both micro and nanocarrier-based vaccine delivery systems have been explored, to act as an effective antigen carrier, to modulate the immune responses, to protect the antigens from a deleterious environment until delivered to the immune cells and to allow a controlled release of antigen [10].

However, due to their small size and large surface-area-to-volume ratio, nanoparticles can interact with biomolecules, both extracellularly and intracellularly [11]. In this context, the investigation of silk proteins as a drug carrier has been widely expanded over the last few years, since sericin and fibroin can be tailored via genetic engineering to contain specific chemical features [12].

However, when engineered to nanosize, nanocarriers may suffer degradation and exhibit toxic biological effects. Biodegraded nanoparticles may accumulate within cells and lead to intracellular changes such as disruption of organelle integrity or gene alternations. In contrast, the physicochemical and biological properties of MCs are easily controlled [13].

1.2 Cell culturing systems with MCs

Conventional routine cell culture with MCs is performed under static conditions in Petri dishes, T-flasks or roller bottles [14]. In spite of being easy to use, disposable and low-cost, these systems require individual handling. Alteration of cell-specific extracellular matrix secretion, loss of specific morphology and phenotype can occur during passaging [15]. Furthermore, controlled environmental parameters and homogeneous diffusion of nutrients, gas–liquid oxygen and metabolites is generally impossible [7, 8]. Thus, these conditions often have low seeding efficiencies.

To overcome these limitations, dynamic culturing in bioreactors has been used for the seeding and proliferation of cells on MCs [16, 17]. The use of bioreactors, such as stirred tank and rotating chamber reactors, has several advantages. These systems are widely used for microbes [18, 19] and mammalian [20, 21] cells culture under monitored and controlled operational parameters (e.g. pH, temperature, oxygen tension, and nutrient supply) up to an industrial scale [8]. Furthermore, bioreactors allow batch and continuous operation [3, 9]. While batch mode operation only provides limited amounts of material, the continuous mode allows a more efficient use of reagents and enhanced reproducibility [22].

However, the ability of the cells to populate the MCs largely depends on their physicochemical properties, such as their surface properties and particle size. Moreover, a narrow crystal size distribution allows a homogeneous cell growth around the particles. Thus, the physicochemical properties of MCs can determine their behaviour in a dynamic culture [3, 5]. MCs core materials can be divided into synthetic or natural polymers. Even though synthetic polymers exhibit good reproducibility and mechanical properties, their cell adhesion and proliferation properties are low. On the other hand, in addition to being easier

and cheaper to obtain, natural polymers show better biological responses [7].

2 Silk MCs

2.1 Silk proteins

Silk proteins represent a unique family of natural fibrous proteins due to their unique properties for the development of MCs [23]. These proteins exhibit non-toxicity, biodegradability, self-assembly, mechanical stability and have a controllable structure [24, 25]. There are several species of insects that produce natural silk, such as silkworms, spiders, scorpions, mites, and flies [26]. The silkworm is a native insect from North China and currently the most used in silk production, being *Bombyx mori,* the most common species of domesticated silkworm [27]. Regarding the type and origin of silk, most of the literature reported studies to use *Bombyx mori* cocoons as silk source [17, 28, 29]. Silk threads from silkworm cocoons consist primarily of two protein components, fibroin, a semi-crystalline fibrillar protein, and sericin, the water-soluble glue-like protein that operates as a binder to maintain the structural integrity of the cocoon [26] (Fig. 1).

Silk fibroin has been used for centuries in the textile industry and for decades in biomaterials, providing stiffness and resistance. Besides providing good mechanical properties and allowing the formation of 3D structures, fibroin is also biocompatible [30]. On the other hand, sericin was traditionally disposed of in silk processing wastewater. More recently, this protein has been reported to improve biocompatibility in vitro and increase cell adhesion and proliferation of several mammalian cell lines [31]. The main advantages and disadvantages of each silk protein are included in Table 1.

2.2 Composite silk-based MCs

Despite their characteristics, silk proteins are usually modified by chemical and physical methods to enhance their properties [32, 33]. A more widespread approach is the combination of silk sericin or fibroin with other materials, forming composites to accommodate a broader spectrum of functional requirements [34].

Silk fibroin-based biomaterials with alginate (Section 3.1), gelatin (Section 3.2), calcium phosphates (Section 3.3) and pullulan (Section 3.4) are the most produced silk MCs. Although MCs are traditionally spherical in order to provide a high surface area to volume ratio, other 3D shapes have been reported [7]. Spherical-like morphology can be challenging to sort or further process. In addition to being directly exposed to surrounding flows and surfaces, the cells attached to spherical MCs are constantly changing locations over time. Moreover, conventional methods to obtain microspheres include emulsification and polymerisation, followed by additional treatments, hindering its continuous production [35, 36]. Thus, other reported works focus on the development of other silk 3D constructs to self-organise into properly differentiated functional cell types. Among them, silk fibroin microparticles with chitosan and collagen (Section 3.4) and silk organoids (Section 3.5) have been recently developed.

Studies on the efficiency of these silk-based MCs and microparticles usually involve seeding with mesenchymal stem cells (MSCs). The therapeutic function of these cells is related to their multi-differentiation potential, which gives these cells the capacity to form bone, cartilage, fat and other multipotent cells [6]. Further, these cells remain at the injury site, secreting trophic and immunomodulatory bioactive factors [37].

Silk proteins are proven to promote cell adhesion, proliferation and differentiation of MSCs [38].

Although there are several studies in which sericin is combined with other inorganic and organic polymers to synthesise different biomaterials such as porous and fibrous scaffolds [39], hydrogels [40], films [41] and microspheres [42], to our knowledge, studies focusing on the development of sericin-based MCs are still inexistent [24], due to its weak mechanical properties and water solubility [43].

Therefore, the present work focuses on reported studies on the synthesis and physicochemical characterisation of silk fibroin-based MCs, on the biological response and cell culturing conditions used, as well as on the potential applications of these biomaterials (Fig. 2).

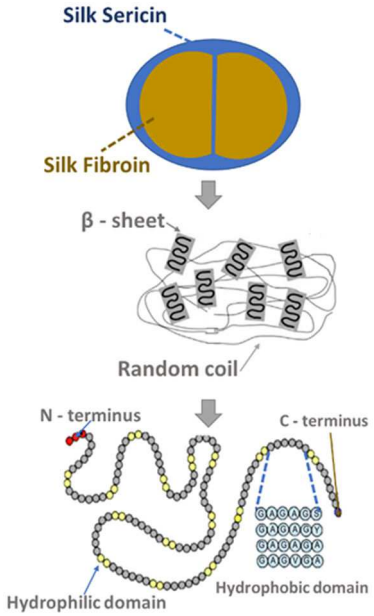

Fig. 1 *Structure of silkworm Bombyx mori silk sericin and fibroin*

Table 1 Summary of the main characteristics of silkworm proteins: silk fibroin and silk sericin main reported advantages and disadvantages

Silk proteins	Main physiological function	Advantages	Disadvantages	References
fibroin	structural integrity	- biocompatibility; - biodegradability; - good oxygen permeability; - mechanical strength; - elasticity; - easily processable; - low cost	- stringent protein isolation and purification protocols; - low osteogenic capacity;	[44–52]
sericin	protection	- biocompatibility; - biodegradability; - moisturising power; - resistance to oxidation, bacteria, and ultraviolet light; - usually discarded during silk processing	- stringent protein isolation and purification protocols; - weak mechanical strength	[53–57]

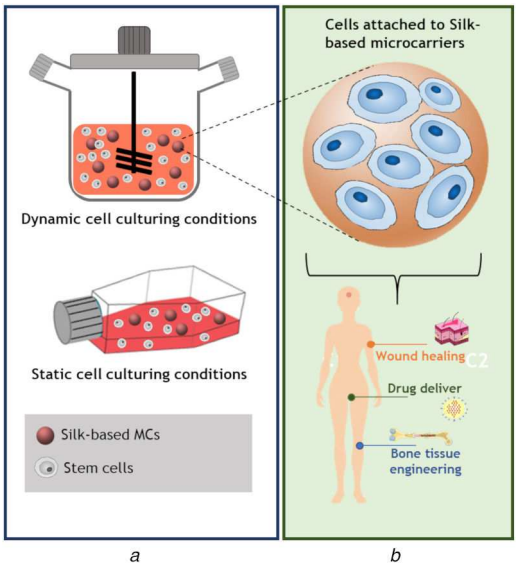

Fig. 2 *Cell culturing systems and its applications in biomedical engineering*
(a) Static and dynamic culturing systems used to produce 3D constructs, ***(b)*** Conventional structure of silk-based MCs reported in the literature and their main applications

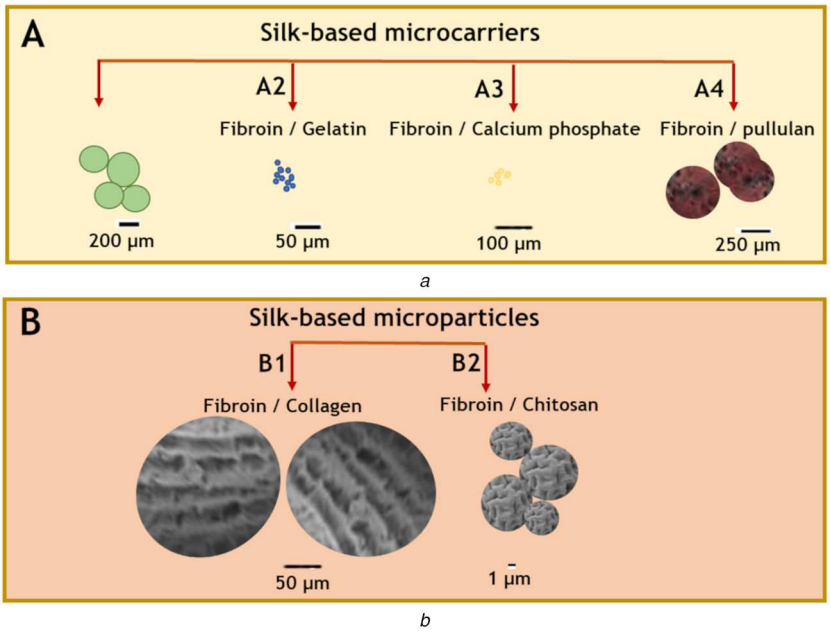

Fig. 3 *Illustration of the conventional structure of*
(a) Silk-based MCs reported in the literature: (A1) fibroin/alginate MCs based on [58], (A2) fibroin/gelatin MCs based on [59], (A3) fibroin/calcium phosphate adapted based on [29] and (A4) fibroin/pullulan based on [60], ***(b)*** Silk-based microparticles: fibroin/collagen (B1) based on [61], fibroin/chitosan (B2) based on [62]

3 Silk fibroin-based MCs

Pure silk fibroin has been used to develop MCs for cell culture under mild processing conditions. In the work of Wang *et al.* [63], fabrication of these materials was achieved using a high voltage electrostatic field, in order to obtain fibroin spherical droplets. The silk spheres were posteriorly subject to freeze-drying, resulting in highly porous biomaterials with diameters ranging from 208.4 to 727.3 µm. The smaller particles (200–300 µm) were more suitable for murine fibroblasts cell line L-929 adhesion and proliferation when compared to larger particles. Fibroblasts, besides being responsible for the production of the extracellular matrix, also play an important role regulating interstitial fluid volume and pressure and wound healing, thus being suitable to study MCs for different biomedical applications [64].

Bessa *et al.* [65, 66] proposed the use of fibroin microparticles for bone tissue engineering (bone-TE). In these works, silk MCs were obtained as a delivery carrier for bone morphogenetic protein-2 (BMP-2), through a coprecipitation method. Spherical particles with a mean size of ~580.0 nm and BMP-2 encapsulation efficiency of 97.7 ± 2.0% were obtained [66]. BMP-2 released from the MCs was able to induce rapid cell differentiation of immortalised cell line C2C12, originally isolated from mouse muscle [67], into osteoblasts and mineralisation. *In vivo* assays on a rat ectopic model showed that after four weeks, new bone was formed and an increase in bone density over time resulted from adding BMP-2 loaded MCs. For non-loaded MCs, bone formation was not detected, thus meaning that fibroin/BMP-2 MCs retain growth factors at the site of the injury for a period of time without being immediately degraded [68].

In addition to pure silk fibroin MCs, different biopolymers in combination with silk have been investigated for MC formulation, in order to improve and tailor both mechanical and biological properties [69, 70] (Fig. 3).

3.1 Silk/alginate MCs

Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed, which is used as a core, giving stability in MCs due to its biocompatibility, low toxicity, low cost, and fast sol–gel transition in contact with divalent cations. However, this polymer is unable to specifically interact with

mammalian cells due to the presence of negative charges and its deficiency of integrin domains [71–73]. Thus, conventional methods to overcome this disadvantage include blending with natural proteins such as silk proteins.

In the reported works [28, 58, 74], the preparation of alginate cores was achieved by dropping alginate droplets in a calcium chloride water solution. Silk/alginate biomaterials were obtained after immersing the cores in a 1.5% w/v silk fibroin solution. Conventionally, these composites are immersed or washed with 96% (v/v) ethanol to induce the conformational transition of silk fibroin from insoluble β-sheet conformation to a water-soluble structure [28, 58, 74]. The biological response of the resulting materials was evaluated after seeding with adipose-derived stem/stromal cells (ASCs). Adipose tissue is one of the most convenient sources of MSCs due to its availability and accessibility [75]. ASCs adhered rapidly to the spherical MCs with an average diameter of 400 μm. After 7 days, the cells were homogeneously distributed and built connections on the biomaterial surface. After 14 days, numerous 3D constructions were created by the interaction between adherent cells. Evaluation of osteogenic, adipogenic, and chondrogenic potential showed that ASCs adhered to the surface of the MCS can produce mineralised matrix and form lipid droplets, indicating that the cells maintained their viability and multipotency [28, 58].

Perteghella *et al.* [58] and Duchi *et al.* [28] followed a conventional static cell seeding process of MSCs in the MCs, whereas, in the work of Perucca Orfei *et al.* [74], cells were co-incubated with MCs in a bioreactor for different time intervals and conditions. In order to make silk fibroin/alginate more stable, the MCs were freeze-dried before optimising the seeding conditions. It was later confirmed that lyophilisation did not affect the MCs structure. Cell density and sample quantity in an oscillating shaker were fixed while the duration of time, stirring speed, dynamic culture and volume of MCs/cell suspension were the variable process parameters. Both adipose-derived stem cells (hASCs) and human bone marrow stem cells (hBMSCs) showed a good cell adhesion rate on the fibroin coated materials. hASCs were able to adhere to the MCs in <2 h, under suitable conditions. In particular, the dynamic seeding of cells provided the best outcomes in comparison with static cultures, in terms of cell adhesion and viability. The best outcome was achieved for the following seeding conditions: 98 min of incubation time, 12.3 rpm of stirring speed, and 401.5 μl volume of MCs/cell suspension-cultured with the intermittent dynamic condition. The last parameter was found to deeply influence the final cell adhesion, proliferation, and differentiation.

3.2 Silk/gelatin MCs

Gelatin, a biocompatible and biodegradable protein obtained from the hydrolysis of collagen, can be easily cross-linked due to its large number of functional groups. Its characteristic RGD sequence (arginine–glycine–aspartic acid) is essential for stable relationships between the cells and the surrounding extracellular matrix, being recognised by integrins and cell surface receptors. Despite its good biological properties, gelatin has poor mechanical properties and is therefore combined with other materials such as silk [76].

Silk composites with 30% gelatin were obtained by cryodestruction in the studies conducted by Arkhipova *et al.* [59, 77]. Morphological evaluation of the synthesized MCs evidenced the formation of an irregular-shaped and porous surface. Increased cell adhesion and proliferation of 3T3 murine fibroblast of silk/gelatin MCs in vitro were confirmed by Arkhipova *et al.* [59] under static culture conditions. Although complete wound healing was observed in the control group and in the fibroin and fibroin/gelatin MCs, the composites promoted regeneration of damaged areas to a greater extend by regenerating muscle tissue and subcutaneous hypodermis [77]. In vivo, subcutaneous administration in mice of silk/gelatin MCs was performed in another study conducted by Arkhipova *et al.* [77], demonstrating that these biomaterials are also suitable for the treatment of deep skin wounds. These MCs increase expression of proinflammatory cytokines, which are the key mediators of inflammation [78].

Spherical and porous MCs for osteogenic tissue engineering were first studied by Luetchfird *et al.* [17]. The MCs were synthesized under dynamic culture conditions using a microfluidic flow system to reproducibly obtain MCs with controlled properties [17]. Gelatin and fibroin solutions were mixed into blends with varying fibroin/gelatin ratios and linked to the inner phase of a T-junction flow-focusing device. The outer phase consisted of oleic acid, methanol and Span 80 to induce β-sheet conformational change in silk fibroin. The inner and outer flow rates were fixed in order to compare the physicochemical characteristics of the different MCs collected (device output) and to generate biomaterials with diameters comparable with commercially available MCs. It was observed that the MCs synthetized, besides having a mean particle diameter in the range of 300–400 μm, also have a narrow size distribution.

Homogeneous MCs were mainly formed in fibroin/gelatin 50:50 and 25:75% w/v, having these blends higher mechanical properties and seeding efficiencies. Contrary to what was expected, pure gelatin materials did not promote rat MSC (rMSC) isolation proliferation. Gelatin swelling resulting from water absorption of the culture medium can occur, reducing the biomaterial mechanical properties and making it unsuitable to stimulate cell adhesion [79]. By adding fibroin, for all conditions studied, cell adhesion and osteogenic proliferation were significantly increased. Contrary to the other reported works on silk/gelatin MCs [59, 77], the system used has the advantage of allowing scale up production of therapeutic cell systems [17].

3.3 Silk/calcium phosphate MCs

Besides natural polymers, inorganic components such as calcium phosphates have also been used to synthetise silk MCs. These compounds are the main components of hard tissues, exhibiting properties of biocompatibility, bioactivity and osteoconductivity [80].

Fibroin MCs modified by calcium phosphate mineralisation have also been studied. In the work of Kotliarova *et al.* [29] and Goncharenko *et al.* [81], porous MCs with the size ranging from 100 to 250 μm were obtained by cryodestruction of the matrices to generate fibroin, fibroin/gelatin, fibroin/calcium phosphate and fibroin/gelatin/calcium phosphate materials. Osteosarcoma cell lines (MG-63) and MSCs cell adhesion occurred for all types of MCs. Cell proliferation was found to be significatively lower for mineralised MCs, probably since substrate mineralisation contributed to the induction of osteogenic differentiation in the absence of inductors, reducing the rate of proliferation [81]. While gelatin can be added to the material in order to promote the cellular response [76], calcium phosphate surface increases the MCs roughness. Although this mechanical factor is not linked to significant changes in cell proliferation, it can promote osteogenic differentiation [82]. Therefore, these fibroin/calcium phosphate MCs can be used to stimulate bone regeneration.

3.4 Silk/pullulan MCs

In addition to the silk bioconjugates covered so far, there is also a work conducted by Aydogdu *et al.* [60] in which fibroin is used as a coating instead of being used as the core of MCs, as conventionally reported. Instead, pullulan, a natural polymer, was used as the main component of the MCs due to its non-toxic, non-immunogenic and biodegradable nature [83].

Pullulan (15%, w/v) was cross-linked by trisodium trimetaphosphate (7.1% w/v), being this homogeneous solution further mixed with calcium carbonate microparticles with a mean size of 20 μm. The final mixture, in combination with biodegradable surfactants, was added to a stirred tank batch reactor with a stirring motor at 500 rpm. To obtain a porous construct, a porogen leaching phase was implemented after the water-in-oil emulsion methodology. The silk coating was obtained via reductive amination, producing particles with an average size of 169.9 ± 45.4 μm. To improve both biological and mechanical properties, pullulan/fibroin MCs were further processed using biomimetic mineralisation by incubation in simulated body fluid (SBF). After 7

days of SBF incubation, sphere-like MCs with ∼150.1 μm were obtained. As expected, higher mechanical properties and cytocompatibility were obtained by increasing the MCs stability and their bioactivity upon treatment with SBF. The in vitro osteogenic potency of the MCs was investigated using SaoS-2 cell line, which displays several osteoblastic features, in static and dynamic culture conditions. Similar cell adhesion was obtained for fibroin-coated MCs and mineralised MCs, in both culture conditions after 1 day of incubation. Additionally, pure pullulan MCs have lower cell adhesion when compared to fibroin/pullulan MC. However, in dynamic conditions, significantly higher cell attachment was observed. Regarding cell proliferation, contrary to what was expected, the overall profiles of cell viability and proliferation were found to be similar for static and dynamic culture conditions. This can be due to the parameters fixed for the dynamic cell culture. According to the review presented by Egger *et al.* [84], a higher cell number in dynamic cultivation conditions is usually obtained for moderate culture mixing speeds.

3.5 Silk/chitosan and silk/collagen microparticles

There are other works in which silk fibroin-based microspheres are developed, though the term MCs is not used. They are based on 3D materials at the microscale and with the same morphology to promote cell adhesion and expansion. The combination of silk fibroin with positive charge polymers, such as chitosan and collagen, to obtain microparticles to apply in mucus or similar physiological environments has been the subject of study [62].

On the one hand, chitosan is a biocompatible polymer that promotes cell adhesion and migration, being thus combined with fibroin [85]. On the other hand, collagen is the most abundant protein present in mammalian tissues, tendons, ligaments, bone and skin and is therefore widely used in biomaterials for biomedical engineering. Besides promoting cell attachment, this natural protein has excellent biocompatible properties and can be easily degraded and resorbed by the body [86]. However, poor physical and mechanical properties, and poor thermal stability, justify combination with fibroin in order to obtain a material with suitable structural integrity [87].

Nimisha *et al.* [61] compared the biological characteristics of fibroin, fibroin/chitosan and fibroin/collagen microparticles. To do so, silk microparticles were obtained by dropping droplets of a fibroin/HFIP (hexafluoroisopropanol) solution in a methanol coagulant bath. The surface of these microparticles was then modified with 1 wt% chitosan or collagen. The physicochemical properties of the three experimental conditions were similar, with pores of the order of 100 mm. However, the coated materials led to an increase in the number of viable MG 63 osteoblast-like cells. Further, cell attachment and viability was higher in fibroin/collagen composites. However, the osteogenic differentiation was better in fibroin/chitosan microparticles.

Chitosan is also widely used in the controlled release of bioactive proteins or peptide drugs. Nevertheless, chitosan microparticles have limitations such as a high initial burst release rate and a short period of sustained-release [88]. Several works focus on the synthesis of fibroin/chitosan to increase the release period, using a spray-drying technique [62, 89] or an emulsification approach [90–92]. The microspheres obtained usually present a smooth [88, 90] or wrinkled surface [90–92] with a diameter that can range from ∼7 to 150 μm [90].

The dissolution of the microparticles can be controlled by adjusting the fibroin/chitosan blend ratio. According to the study of Cheerarot and Baimark [91], increasing the chitosan content in the blend led to a further decrease in the dissolution percentage. Adding different components is also a route to obtain different release periods. In the work of Chung *et al.* [62], an antibiotic (tetracycline-Hcl) was encapsulated into silk/chitosan and silk/chitosan/tri-polyphosphate (TPP). The release period of pure fibroin (two days) was prolonged to four and ten days for fibroin/chitosan (10/4.0 wt%) and silk/chitosan/TPP (0.05%) microparticles, respectively [62]. A lower initial burst release and a prolonged cumulative release of up to 21 days were achieved in [90], by using genipin as a cross-linking agent to produce fibroin/chitosan microparticles (1:1%wt) to encapsulate bovine serum albumin (BSA). It was also found that by using 0.05 g of genipin, particles with the most uniform particle-size distribution were obtained. In addition to the in vitro release studies conducted in the reviewed papers, Hu *et al.* [92] confirmed the potential of these composites in vivo. The microparticles were orally administered to rats, and their blood concentration was measured at specific time intervals.

Fibroin/chitosan composites have also been explored in the work of Yang *et al.* [93] as possible cardiac patches. Microspheres with particle sizes ranging from 70 to 147 μm, obtained through spray-drying, led to a better relative growth rate of rMSCs than pure chitosan. In addition, the cardiomyogenic differentiations of rMSCs on the silk-based patches in vitro were further enhanced by adding hyaluronic acid.

3.6 Silk organoids

Other innovative silk 3D constructs that have been developed to promote cell adhesion and proliferation are organoids. These structures form organ-like tissues that mimic natural organs, structurally and functionally in vitro. Lyophilised silk scaffolds were formed and seeded with kidney progenitors [94]. The main limitation highlighted in these structures is the lack of in vivo-like functionality, such as vascularisation. For tissue to grow beyond 100–200 μm, expanding into a microtissue, new blood-vessel formation is required to supply the individual cells with nutrients and oxygen. Otherwise, nutrient deficiencies, hypoxia, on-uniform cell differentiation and integration can occur in the tissue [95].

Potential strategies for enabling organoid vascularisation include the use of silk fibroin in laser photo-ablation methodologies [96]. Gupta *et al.* [97] evaluated the capacity of silk to serve as an organoid to form kidney tissue from primary cells and human-induced pluripotent stem cells (iPSCs). This trending silk technology was found to be well suited to support iPSCs growth and differentiation into kidney tissue. However, the proliferation of stromal cells within the graft and tissue organisation stills presents a challenge to overcome [97].

According to the final application, different types of silk composites and cell types can be used, as summarised in Table 2.

4 Future perspectives

MCs offer an environment that resembles biological systems, where cells can proliferate, maintain their cell phenotype and expand on a larger scale. Due to these properties, MC systems have the potential to produce 3D microtissues, which can be used to regenerate and restore damaged tissue. Thus, MCs have a huge potential in regenerative medicine [7].

MCs are a recent technology, being most of the reported work from the last 5 years. Despite the growing interest in this topic, there are still several aspects to be studied and explored.

4.1 Exploring silk-based MCs for biomedical applications

Although silk-sericin MCs have not yet been reported, this type of biomaterial has a great interest in biomedical engineering. Sericin can be easily incorporated in the reviewed MCs through the coating, in order to improve the biological activity of MCs [24]. Even though most silk-based MCs use fibroin as the core material, the works carried out with pullulan and alginate (Sections 3.1 and 3.4) demonstrates that other materials can be used to give integrity to the material and that silk protein can be incorporated through different methodologies. An increasing number of studies have shown that sericin improves cell adhesion and proliferation when used as an organic matrix or a medium for cell growth [98–100]. Moreover, sericin can be used as serum-free media, substituting foetal bovine serum, thus being recovered for biomedical applications [101, 102]. These properties can be useful to maintain the integrity, structure, and intrinsic function of microtissues developed from MCs, allowing the creation of stocks for later use or even opening new avenues for the transportation of synthetic tissue substitutes to remote locations. Nevertheless, recently proposed technologies for enhancing the stability of sericin, such

Table 2 Silk-based 3D structures reviewed, cell types used and potential applications

Biomaterial	Synthesis methods	Cell type used	Potential applications	References
silk fibroin MCs	droplet production	murine fibroblasts L-929	wound healing	[63]
	co-precipitation	C2C12 cell line	drug delivery system for TE applications	[65, 66]
silk fibroin/alginate MCs	droplet production followed by fibroin coating	hASCs hBMSCs	cell delivery for advanced therapy and regenerative medicine of bone and cartilage diseases or defects	[28, 58, 74]
silk fibroin/gelatin MCs	freezing thawing	3T3 murine fibroblast	wound healing	[59, 77]
	mixing	rMSC	bone TE	[17]
silk fibroin/calcium phosphate MCs	freezing thawing	MG-63 MSCs	bone-TE	[29, 81]
silk fibroin/pullulan MCs	water-in-oil emulsion	SaoS-2 cell line	bone-TE	[60]
silk fibroin/chitosan microparticles	spray-drying	rMSCs	cardiac patches	[93]
	—	—	drug delivery systems	[62, 89]
	emulsification	—		[90–92]
silk fibroin/Collagen microparticles	droplet production followed by coating	MG 63 osteoblast-like cell line	bone-TE (load-bearing osteo-regenerative applications).	[61]
silk organoids	freeze-drying	iPSCs	regenerative medicine – kidney tissue	[97]

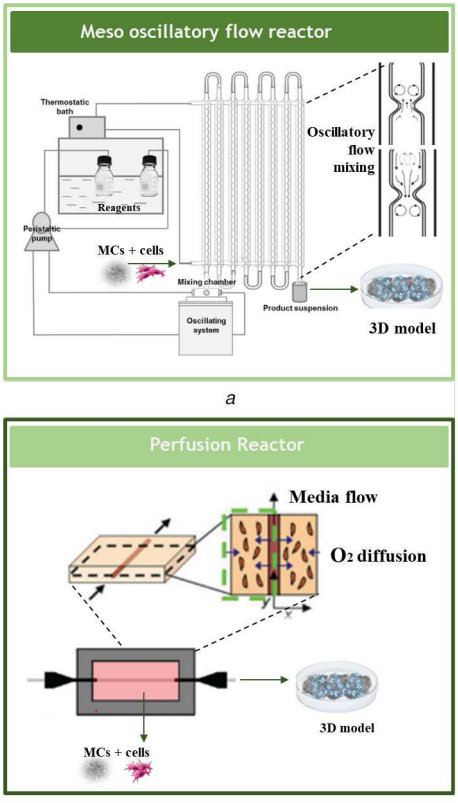

Fig. 4 *Illustration of the implementation of recently developed bioreactors in the production of an MC-based 3D construct*
(a) Meso-OFR (image adapted from [103]), ***(b)*** Perfusion bioreactor (image adapted from [104, 105])

as enzymatic cross-linking (WO/2018/011732), might open room for the development of viable MCs.

Additionally, specific growth factors or extracellular matrix proteins, besides BMP, can be included in silk-based MCs to further aid cell adhesion and expansion. Hence, these 3D materials can serve a dual role as both delivery systems of bioactive factors and scaffolds for cell proliferation and differentiation. Moreover, other components that can be used in the development of new silk-based MCs for different biomedical applications are dextran, cellulose, plastic or glass [106].

4.2 Dynamic culture systems to generate silk-based MCs

Although the use of bioreactors to perform cell seeding on MCs is a promising way to mimic and simulate the biological environment in order to obtain 3D constructs, studies of fibroin-based MCs with dynamic cell culture are still scarce [60–74].

In addition, conventional spinner-flask and stirred tank bioreactors to have limitations related to mass transfer phenomena that can affect both the producibility and the product characteristics [7]. Since the characteristics of MCs (e.g. chemical composition, surface topography, charge density, particle size etc.) are intrinsically related to their ability to promote cell adhesion and proliferation, it is important to find solutions to these limitations [7, 8, 107]. Further, for clinical applications, it is also important to obtain a fully functional microtissue, overcoming the main limitation of the silk organoids reported: lack of vascularisation [97, 108]. Employing these systems is also related with other critical issues such as the standardisation of bioprocesses and limited upscaling [14].

In that regard, oscillatory flow reactors (OFRs) are an example of a 'technology ready to deliver' [109, 110]. In particular, a recently developed meso-OFR (meso-OFR) [110] has been shown as a promising tool in multiphase systems, namely by promoting a significant increase in the intensification of mixing leading to the formation of materials with controlled properties [111, 112]. The device consists of a glass tube at the mesoscale provided with smooth periodic constrictions (SPCs) operating under oscillatory flow mixing. The intensity of the mixture is controlled by the frequency and amplitude of the fluid oscillation [110, 113, 114]. This reactor has been successfully used in the precipitation of non-toxic, pure hydroxyapatite and hydroxyapatite/silk sericin materials with improved cell viability when compared to a commercially available hydroxyapatite powder [43]. Moreover, meso-OFR has been successfully implemented in the growth of fungi cells [113]. Thereby, the OFR has the potential for both the synthesis of MCs and for posterior tissue maturation. Further, the scale of these devices can be easily extended and the devices can operate within a batch and continuous flow regimes (Fig. 4*a*) [115]. Large-scale culture technology is crucial in MCs and organoid technology to obtain the number of stem cells required for clinical application [6, 97].

Post-processing of the 3D structure can also be achieved using a perfusion bioreactor. These reactors improve mass transfer not only on the external surfaces of tissue scaffolds, but also around internal areas, stimulating tissue growth and maturation in a homogeneous manner [116, 117] In the work of Zhang *et al.* [118] and Grasman *et al.* [104] a perfection reactor was used for the integration of vascular cells into silk scaffolds (Fig. 4*b*).

Innovative technologies covered in this review, which promote cell adhesion and expansion, such as bioprinting and conventional

chemical processing methodologies, can be further complemented with the highlighted bioreactors.

Despite being a well-established method to assemble cells and specific extracellular matrix within a 3D construct, the development of bioprinted skin-like constructs still faces several challenges, including the promotion of critical cell–cell interactions and signal communications. The selection of suitable bioinks and of in vitro culture systems that allow for cell expansion are among the methods used to improve tissue authenticity over conventional tissue equivalents [106]. In this context, silk-based MCs are a developing technology used to promote a high-yield culture of anchorage-dependent cells providing appropriate microenvironments for cell interaction in vitro and allowing the standardisation of bioprocesses. Fibroin MCs with tailored properties, can be obtained using an OFR and loaded in the bioink to obtain a specific 3D microtissue. The perfusion reactor can be used as a final step for the development and maturation of vascular and neuronal tissues.

5 Conclusions

MCs systems have a porous microstructure, in order to allow cells to attach and grow. These 3D constructs conventionally adopt a spherical morphology; however, other morphologies have been explored. Besides providing a high surface area to volume ratio for maximising cell number during cell expansion, MCs allow efficient gas–liquid oxygen exchanges, being cost-effective and space-saving. In this context, the scientific interest in silk-based composites has considerably increased in recent years. Silk fibroin combines biological properties, such as biodegradability and non-toxicity, with mechanical strength and stability. Silk fibroin is the most widely studied silk protein.

In short, the herein reviewed literature highlights the diversity of potential biomedical applications of MCs and other recent 3D silk-based structures. Despite the advances made in the last decade, there are still several studies that need to be conducted. The use of silk sericin in MC systems, as well as the development of further studies on the behaviour of MCs in conventional and new biological reactors, is among the future perspectives discussed in this paper.

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