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Oxygen permeability of silk fibroin membranes: A critical review and personal perspective

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Abstract

Membranes made of *Bombyx mori* silk fibroin are currently used as substrata for growing cells with an aim to generate biomaterial-cells constructs for tissue engineering applications. Although the oxygen transport characteristics of the membranes are important in these applications, there is limited work reported on the oxygen permeability of silk fibroin. This review discusses critically the existing literature and attempts to explain the scarcity of data on this topic.

Introduction

Fibroin is the main proteinaceous component of the silk thread produced by larvae of the domesticated silk moth (*Bombyx mori*) and by the wild species of silk moth. It is a naturally designed polypeptidic composite belonging to the group of fibrous proteins, and is characterized by highly repetitive amino acid sequences leading to a predominantly homogeneous secondary structure, which is responsible for the exceptional functional performance of the silk thread.

There is a substantial body of literature [1–12] regarding the applications of silk fibroin, especially of *B. mori* silk fibroin (henceforth, BMSF), in tissue engineering and regenerative medicine. Indeed, BMSF displays features that make it attractive as a material for creating substrata (membranes, scaffolds) for growing cells with an aim to regenerate tissues. Such features include acceptable mechanical strength, suitable permeability for the nutrient/waste cellular exchange, protracted biodegradability, and transparency when required. The researchers at Queensland Eye Institute in Brisbane, Australia, were the first to propose and evaluate BMSF as a membranous substratum for growing the cells of the eye (corneal, retinal) [5,13–18], a topic that has since been investigated by other groups too [19–24]. In ophthalmic tissue engineering, where BMSF is mostly applied in the form of membranes, the transport properties of such membranes become an important issue.

To date, the oxygen permeability of the BMSF has been much less investigated, but is more controversial, than its other properties. Sufficiently high oxygen permeability is portentous for applications involving BMSF as implanted membrane-cells constructs, where the presence of oxygen and nutrients are relevant to the growth and proliferation of cells. However, there have been few investigators involved in measuring and/or improving the oxygen permeability of BMSF membranes. This review analyzes critically such efforts.

Early investigations

Almost without exception, all publications dealing with the biomedical applications of BMSF specifically mention in their introductory sections that BMSF is permeable to oxygen. Many of them include also the statement that BMSF can be used as material for manufacturing contact lenses, where we know that the permeability to oxygen is paramount. Some of these reports do not cite any literature to support such assertions, while most of them cite as supporting references the publications from Minoura's group in Tsukuba, Japan. Indeed, this group is widely recognized as being the first to introduce and evaluate BMSF as a biomaterial [25–27]. They have used in-house developed electrochemical method and instrumentation [28] to measure the oxygen permeability (henceforth, ***P***) of hydrated BMSF (i.e. as a hydrogel), and concluded that the measured values place BMSF at the same level as the materials for contact lenses [25]. In reality, the highest value that they measured around physiological temperatures was around 10 Barrer for those BMSF films that were treated for the shortest time in aqueous methanol (to achieve physical crosslinking and ensuing gelation). The equilibrium water content of the membranes was between 20 and 40% and decreased with increasing duration of immersion in methanol. The measurement of ***P*** was done at 34 °C "in wet membranes", but there is no indication as to how the dehydration was avoided during measurements. As a note, the "Barrer" is the accepted name for the non-SI oxygen permeability unit, and has been defined as below:

$$1\ \text{Barrer} = 10^{-10}(\text{cm}^3\text{O}_2(STP)\cdot\text{cm})/(\text{cm}^2\cdot\text{s}\cdot\text{cmHg}),\ \text{or}$$

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1 Barrer=10^{-11}(cm^3O_2(*STP*)·cm)/(cm^2·s·mmHg),

where "STP" stands for "standard temperature and pressure".

In a more detailed study [29], Minoura and colleagues reported values for ***P*** of BMSF between about 3 and 11 Barrer and investigated in more detail the effect of the immersion time in methanol. The shortest the immersion was, the higher were both the water content and permeability. A "permeability coefficient" $\boldsymbol{P}_a$ has been also introduced by these authors to represent the oxygen permeability of totally amorphous fibroin. For the BMSF that was subjected to the shortest immersion in methanol, a $\boldsymbol{P}_a$ ~3 Barrer has been estimated.

While I agree that the oxygen permeability values measured by Minoura and colleagues might have been comparable to the values of ***P*** (or ***Dk*** in contact lens terminology) of the daily wear contact lenses available *at that time* on the market, by the current standards 10 Barrer is definitely an unacceptably low value. Such low oxygen permeability cannot allow sufficient oxygen to reach the ocular surface and assure the normal metabolism of the cornea during contact lens wear [30–32]. For the contemporary contact lenses, values of ***P*** for daily wear are commonly over 60 Barrer, while for extended wear is over 100 Barrer and can be as high as 140 Barrer and beyond. To put into a larger perspective, ***P*** for polydimethylsiloxane is 600 Barrer [33], and for poly (methyl methacrylate) is 0.5 Barrer [32].

Less oxygen allowed to pass across a substratum into the physiological environment of growing cells can be detrimental to the development of efficacious membrane-based fibroin-cell constructs to be used in ophthalmic tissue engineering. Clearly, I cannot share some investigators' enthusiasm triggered by a ***P*** around 10 Barrer for the BMSF membranes. If nothing else, such a value denotes poor oxygen permeability that can only lead to insufficient oxygen available for the cellular metabolism.

Further studies on silk fibroin oxygen permeability

The blending of BMSF with other polymeric materials has always been an alternative strategy to improve its properties. A group at Seoul National University has evaluated blends of BMSF with chitosan [34], with a view to use such membranes as artificial skin and wound dressings. The oxygen permeability was measured in a custom-made two-compartment diffusion cell equipped with an oxygen sensor. The value of ***P*** for BMSF alone was around 0.25 Barrer, while for the 50:50 blend was around 0.58 Barrer. These surprisingly low values indicated that practically the materials do not allow oxygen transport, which has not deterred the authors from stating that the blends "showed very high oxygen permeability"! We could suspect that the investigators might have become confused regarding the definition and handling of the units for permeability. It is not clear how the evaporation of water from membranes (estimated to have an initial water content of 33% on hydrated basis) has been prevented during measurements. Nonetheless, these results are at odds with all other values reported for BMSF, and I doubt their factuality. It is also hard to believe that if we mix two different materials that each have poor oxygen permeability, the result is a mixture that acquires a permeability higher than any of its components.

A more recent study [35] has been carried out at Tufts University in Boston, USA, which houses the world's premier centre for silk research. They have investigated the effects of water annealing and of treatment with aqueous methanol on some characteristics of BMSF membranes including the oxygen permeability. For measurements, a commercially available oxygen permeation analyzer was used to provide, in the first instance, the values for the oxygen transmission rate (OTR). These values were then converted into ***P*** values. During measurements, the relative ambient humidity was maintained at two values, 50% and 80% respectively. It was found that the oxygen permeability of BMSF membranes treated with methanol was higher that than that of the water-annealed membranes, and that both relative humidity and duration of treatments had a marked effect on the values of measured ***P***. It appears that the water content of the water-annealed BMSF films was nil (linear swelling ratio *Q*=1), while the methanol-treated films retained water (*Q*=1.6); in the absence of numerical data for the densities of dry and hydrated BMSF, the value of *Q* cannot be converted into percentage water content. For the water-annealed films, ***P*** was between 0 and 1.8 Barrer, while for the methanol-treated ones was between 0.25 and ~5 Barrer. The differences have been attributed to changes in the secondary structure of fibroin caused by the mentioned treatments. Water annealing induced a more densely packed β-sheet conformation as compared to the less ordered packing induced by the treatment with methanol. These experiments, which appear to have been properly conducted, proved unequivocally that the oxygen permeability of BMSF is low. In a positive aftermath, the same team reported recently [36] the advantages that such low permeability can offer when BMSF is used to coat perishable food, e.g. fruits and vegetables, which require an optimal preservation of freshness during storage. Depletion of oxygen reduces metabolic activity and the ensuing decay of fruits or vegetables, thus enhancing their shelf life.

Another confirmation of the inherently low oxygen permeability of BMSF has been provided in a master degree thesis [37] presented at the University of Waterloo, Canada. A custom-made permeation cell setup has been used for measuring the permeation of common gases (O_2, N_2, CO_2) through BMSF membranes. The water content in the membranes was expressed as a degree of swelling of 473%, which is equivalent to around 80% when expressed as water content on hydrated basis. The drying of membranes during measurements was prevented by purging the permeation cell with a stream of humidified oxygen, but we do not know how effective this procedure was. Oxygen permeability was found to be 5 Barrer.

Silk permeability in ophthalmic tissue engineering applications

As a membranous substratum for cell growth, BMSF is expected to have properties superior to the amniotic membrane (henceforth, AM). Transplantation of AM is currently the foundation of the main surgical strategies employed in the management of ocular surface diseases [15,38]. Therefore, a comparison between their oxygen permeabilities would be greatly significant.

It appears that there is only one reported estimation of the oxygen permeability of AM [39]. Regrettably, the authors chose to calculate the value of ***P*** using an equation, rather than measuring it experimentally. Indeed, equations of the form $\boldsymbol{P}=Ae^{BW}$ are available for hydrogels, such as Fatt equation [40] where A=2 and B=0.0411, or Morgan-Efron equation [30,41] where A=1.67 and B=0.0379. In these equations, *W* is the equilibrium water content at room temperature of the hydrogel material, *e* is the base of the natural logarithms, while A and B are constants determined experimentally from the measured *W* and ***P*** of common synthetic hydrogels used to manufacture contact lenses. But here, beside the inherent drawbacks related to the use of such equations as discussed by Tighe and Mann [42], we have a problem: I believe that to use this type of equation for calculating the oxygen permeability of AM is not justified. First, the equations can be applied only to synthetic

polymeric hydrogels (mostly carbon-backbone polymers), as they have been developed based precisely on the characteristics of such materials as measured experimentally. The structure of synthetic hydrogels is vastly different from that of biological tissues or biopolymers such as silk fibroin. There should be little expectation that a biological hydrogel that was designed by nature to fulfil a complex evolutionary task would display the same transport properties as a synthetic material only because they both may have coincidentally the same water content. Second, the authors used, inexplicably, a wrong equation where A=2.667 [39], further stating that this was done according the international standard ISO 9913-1. This is incorrect: the standard [43] clearly recommends the use of Fatt equation (A=2); the value of A=2.667 cannot be found in any other equation and obviously is erroneous. In addition, the same standard recommends that equation should be applied only to the hydrogel designated as material for normalization, poly (2-hydroxyethyl methacrylate) in this case. Moreover, standard ISO 9913-1 has been long withdrawn and superseded by ISO-18369-4 [44], which does not include the use of such equations. The value ***P***~143 Barrer as calculated for the amniotic membrane by these authors [39] is not based on experimental methods, does not appear to have a scientific foundation, and therefore cannot be considered as a true value of the permeability. In addition, in the hydrogels where water is the dominant carrier for the transport of oxygen molecules through material, there is a limiting value of 100 Barrer, which is the theoretical value for pure water (i.e., for a hypothetical material with the water content of 100%) [42]. This aspect alone would invalidate the value reported in this study if water contributes to the oxygen transport mechanism, a possibility that should not be refuted offhand.

Following a similar pathway, other investigators have reported [45] the calculation of oxygen permeability for "four varieties of silk films", without disclosing the nature of samples. It was indicated that their calculation was based on *W* and done according to an international standard, which suggests that the same procedure [39] has been employed, although it was not stated which equation has been used for the calculation of ***P***. As expected, the higher was the water content, the higher were the values for ***P***. For two of the sample groups, ***P***~100 Barrer was calculated, while for the other two ***P***=14 Barrer and, respectively, ***P***=27 Barrer were obtained. Being a conference abstract, no other details of samples and measurements were available, but obviously this report is rather misleading.

Permeability of fibroin-based blends

In addition to the report discussed above regarding BMSF blends [34], the literature search indicated that a group at Chiang Mai University in Thailand have carried out oxygen permeability measurements for systems containing BMSF as a component [46,47]. A rather rudimentary procedure, the differential pressure (manometric) method, has been used to measure ***P***, which was accordingly expressed in percent ratio between two differential readings on the U-tube of the manometer scale. Virtually, this was a non-dimensional unit (cm/cm), not convertible into Barrer or other conventional units. Blends containing no more than 2% wt/vol BMSF with poly (vinyl alcohol) and rice starch have been studied [46]. OTR values have been also measured in a commercially available device, but the results were not converted into units for ***P***. Both ***P*** and OTR values indicated that oxygen permeability was the highest at 2% wt/vol BMSF. If OTR values are compared with those reported elsewhere for BMSF [35], the inescapable conclusion would be that the oxygen transport through the blended membranes is extremely low. The same group also investigated [47] blends consisting of 5% wt/vol BMSF, rice starch and trisodium trimetaphosphate; the last component was added as a crosslinking agent for starch. In addition, the materials were rendered porous by the freeze-drying process. As OTR has not been measured in this study, a discussion on ***P*** values based on simple readings on a manometric scale is meaningless, although the observed trend of ***P*** to increase with increasing porosity is plausible. However, whether or not the levels attained would be physiologically suitable cannot be asserted from this study.

Effect of porosity

Further investigations on the role of porosity on the oxygen permeability have been carried out by the same group at Chiang Mai University, this time using BMSF alone [48]. Porosity has been induced by adding poly(ethylene glycol) (henceforth, PEG) as a porogen, a well-known method. In this study, PEG with a molecular mass of 400 kDa has been used. Other membranes were made by the chemical crosslinking of BMSF with glutaraldehyde, a chemical compound that is *not* known as a porogen. The authors justified the use of chemical crosslinking as a tool to induce pores in BMSF by an assumed ability of glutaraldehyde "to create more empty spaces within the membrane", a statement suggesting that they may have a rather incomplete understanding of the mechanism of the crosslinking process in polymers. ***P*** was expressed in percent ratio read on a manometric scale. When it came to the relation between porosity and ***P***, these authors have found that both properties presented maxima at 40% wt PEG and at 3% wt glutaraldehyde. The drops in permeability recorded prior and after the maxima have been clumsily explained by "more crosslinking between PEG and SF chain when they come together" (?) and, respectively, by the fact that "the membranes become more dense due to extensive cross-linking". Neither explanation is acceptable, and the results of this study, as well as of the other reports coming form the same group [46,47] do not contribute to a better understanding of the oxygen transport through the BMSF membranes.

Summary and conclusions

Table 1 presents a summary of published data on the oxygen permeability of BMSF. The table contains only those data that can be considered reliable and mutually comparable.

The paucity of reported data reflects the difficulties related to the estimation of oxygen permeability of BMSF, rather than being caused by lack of importance of, or lack of interest for this aspect of silk research. The problems commonly affecting availability, accuracy and reproducibility of information on this topic are discussed below.

a) Variability of the procedures and instrumentation from one laboratory to another: virtually, there are not two identical methods used in the published reports, although ISO 18369-4 [44] clearly recommends the *polarographic* method for all types of materials (including hydrogels), and the *coulometric* method for non-hydrogel materials.

Table. Published values for the oxygen permeability of *Bombyx mori* silk fibroin members

Reported values P (Barrer)	Method/instrument	Reference (year)
3–11	Electrochemical (in-house setup)	[25] [29] (1990)
0.25	Diffusion cell + oxygen sensor	[34] (2001)
0–1.8[a] 0.25–5[b]	OTR analyzer	[35] (2010)
5	Permeation cell + flowmeter	[37] (2013)

[a]Water-annealed membranes
[b]Methanol-treated membranes

b) The instruments available commercially for measuring oxygen permeability are too costly for most of the (usually) small silk research laboratories.

c) There may be variability in the protocols to obtain silk fibroin membranes. Although similar, slight differences between them can affect significantly the structure and properties of regenerated fibroin.

d) The use of disproportionately large range of units different from the Barrer, sometimes bizarre hybrids of SI, imperial and US units, which makes the comparison between the results of the measurements performed in various laboratories very difficult, if not impossible.

e) The problem above is due in part to the use in some laboratories of unsuitable methods to measure ***P***. Generally, such methods are associated with the need to introduce improvised units that are neither convertible into Barrer, nor interpretable as such. Moreover, when contact lens hydrogels with a known ***P*** (provided in Barrer by the manufacturers) are subjected to measurements in makeshift setups, the results do not always match the values measured in proper devices and using standardized methods.

From this review, we should conclude that silk fibroin membranes display poor oxygen transport characteristics, regardless of preparation or measurement protocols. However, there is a distinct possibility that the samples of BMSF in the reviewed studies were substantially dehydrated during the permeability measurements. If we accept, at least in part, that the water content governs the oxygen transport in biopolymers, as it does in the carbon-backbone synthetic polymeric hydrogels, then we may consider that the values of ***P*** reported so far for BMSF could have been affected by incidental dehydration of samples.

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