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Controlled release of cytokines using silk-biomaterials for macrophage polarization

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ABSTRACT

Polarization of macrophages into an inflammatory (M1) or anti-inflammatory (M2) phenotype is important for clearing pathogens and wound repair, however chronic activation of either type of macrophage has been implicated in several diseases. Methods to locally control the polarization of macrophages is of great interest for biomedical implants and tissue engineering. To that end, silk protein was used to form biopolymer films that release either IFN-γ or IL-4 to control the polarization of macrophages. Modulation of the solubility of the silk films through regulation of β-sheet (crystalline) content enabled a short-term release (4–8 h) of either cytokine, with smaller amounts released out to 24 h. Altering the solubility of the films was accomplished by varying the time that the films were exposed to water vapor. The released IFN-γ or IL-4 induced polarization of THP-1 derived macrophages into the M1 or M2 phenotypes, respectively. The silk biomaterials were able to release enough IFN-γ or IL-4 to repolarize the macrophage from M1 to M2 and vice versa, demonstrating the well-established plasticity of macrophages. High β-sheet content films that are not soluble and do not release the trapped cytokines were also able to polarize macrophages that adhered to the surface through degradation of the silk protein. Chemically conjugating IFN-γ to silk films through disulfide bonds allowed for longer-term release to 10 days. The release of covalently attached IFN-γ from the films was also able to polarize M1 macrophages *in vitro*. Thus, the strategy described here offers new approaches to utilizing biomaterials for directing the polarization of macrophages.

1. Introduction

The innate immune system is the first line of defense against pathogens upon infection or wounding. Macrophages clear dead cell debris from a wound, help fight pathogens, and present pathogen-associated antigens to T-cells to activate the adaptive immune response. While macrophages are extremely important for immune defense and wound healing, chronic activation of different types of macrophages has been associated with several diseases, including atherosclerosis [1,2], insulin resistance or metabolic syndrome [3–5], and cancer [6]. Treating these diseases by targeting macrophages systemically is not ideal, as the treatment can impair the immune system as a whole. Localized delivery of treatments for chronic macrophage activation is therefore highly desired. Furthermore, implanted devices or tissue engineered constructs can be functionally hampered by the foreign body response, which involves macrophage-derived giant cell formation and development of granulation tissue surrounding the implants. A film or thin coating on the device that can control macrophage response would be highly beneficial.

Acute inflammation occurs in a wound or at an implant site just after the deposition of fibrinogen and blood clotting. Following neutrophil infiltration monocytes are recruited to the site of the wound within the first 24 h to start clearing away dead cell debris and phagocytose bacteria that may have entered the wound [7,8]. As the monocytes infiltrate the wound site they begin to

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differentiate into macrophages [9]. Once at the wound site macrophages polarize into specialized cells for different purposes. Interferon-gamma (IFN-γ) is a cytokine that activates macrophages to polarize along the "classical pathway," which generates inflammatory macrophages, termed M1 macrophage due to the concurrent emergence of type 1 helper T-cells (Th1) [10]. M1 macrophages incite further inflammation by secreting cytokines such as interleukin-1β (IL-1β), IL-6, IL-12 and tumor necrosis factor alpha (TNF-α) [11]. Several markers of M1 macrophage differentiation have been found including C–C chemokine receptor type-7 (CCR7) [12]. These $CCR7^+$ M1 macrophages help to defend against bacterial infections, contribute to phagocytosis of dead cells, and recruit Th1 cells to the site of the infection. Diseases associated with chronic activation of M1 macrophages include atherosclerosis [2], obesity [13,14], and inflammatory bowel disease [15,16].

Following the acute inflammatory phase, M2 macrophages emerge at the wound site, corresponding to the emergence of Th2 cells [17]. M2 macrophages are considered anti-inflammatory because they secrete IL-10, cytokine ligand 13 (CCL13), and transforming growth factor-beta (TGF-β), which promote tissue regeneration [18,19]. The M2 macrophages are polarized through the "alternative pathway" by activation of the IL-4 receptor with either IL-4 or IL-13 [20]. The M2 macrophages are commonly delineated from M1 macrophages by the expression of the mannose receptor CD206 [20], as well as CD163 and CD209 [21–24]. M2 macrophages have been implicated in promoting tissue regeneration and proliferation [25]. In addition, tumor associated macrophages (TAM) are believed to take on M2-like characteristics, including the M2 marker CD206, as they promote tumor progression [26–28].

One of the interesting properties of the different macrophage phenotypes is their plasticity. The ability for an M1 macrophage to become M2 or vice versa has been observed *in vitro* and *in vivo* [2,29–31]. This leads to the possibility that macrophages can be converted using implantable biomaterials to control a specific disease. For instance, conversion of M1 macrophages that are found abundantly in atherosclerotic plaques into M2 macrophages might result in a reduction of plaque obstruction of blood flow by inducing tissue remodeling surrounding the blood vessel. Similarly, conversion of TAMs to M1 macrophages may allow them to better attack the tumors rather than aiding in their proliferation. Materials that can locally release cytokines to differentiate macrophages without systemically affecting the immune system could be very beneficial for the treatment of the macrophage associated diseases.

Recently, decellularized bone scaffolds with IL-4 bound via biotin-streptavidin interactions were used to promote vascular genesis *in vivo* and to control macrophage polarization *in vitro* [32]. That study demonstrates the potential for cytokine manipulation of host macrophages responding to implanted scaffolds. Future materials that can be coated onto any scaffold or medical device and that can release cytokines for macrophage control may have broad applications and implications for the medical industry.

Silk fibroin is a protein derived from the cocoons of the *Bombyx mori* silk worms and has been used for centuries as a suturing material. Silk has more recently been used as a versatile material for other biomedical purposes such as for fabricating scaffolds for cells, and therapeutics [33,34], and for localized drug delivery into wounds [35–37]. Silk fibers can be solubilized and processed into biomaterials that have an advantage over other commonly used implantable materials because they have the ability to stabilize drugs and proteins [38], have a slow and controllable degradation rate, and the degradation products are non-toxic [39,40].

The goal of the present study was to use thin layers of silk protein as films to modulate the release of cytokines to control macrophage responses. In the long run this approach is anticipated to help with the incorporation of tissue engineered constructs and medical device implants into the body and well as improving wound healing. Silk films were utilized in the study as they can be tailored to sustain the release of cytokines and also be processed in aqueous, ambient conditions, to preserve the functionality of the cytokines for these types of *in vitro* studies. The goal to control the polarization of macrophages would have value in fundamental *in vitro* inquiries of macrophage functions, as well as in chronic macrophage associated diseases. If successful, these approaches could be useful for treating a number of diseases from atherosclerosis to cancer by converting the M1 into M2 or vice versa.

2. Materials and methods

2.1. Silk degumming and dissolving

Silkworm cocoons from *B. mori* were purchased from Tajima Shoji Co., LTD (Yokohama, Japan) and are processed into pure silk fibroin solution as previously described [41]. Briefly, cocoons are cut into small pieces and placed into boiling 0.02 mM Na_2CO_3 for 30 min. After boiling, the fibers are washed in deionized water and left to dry in a fume hood overnight. The next day a solution of 9.3 M LiBr is added to the silk fibers at a ratio of 1:4 weight (g) to volume (mL) and then allowed to dissolve at 60 °C for 4 h. The silk solution is then dialyzed against 4 L deionized water for 3 days, changing the water 3 times per day for a total of 7 changes. At that point the silk fibroin is centrifuged at 4000 × g to remove particulate matter and is ready for experiments. To determine the concentration of the silk solution, 1 mL of solution is added to a weigh boat and weighed before and after drying in a convection oven at 60 °C overnight.

2.2. Silk casting and water annealing

To create molds for the silk films to be cast onto, polydimethyl siloxane (PDMS) (GE Plastics, Pittsfield, MA) was cast into a petri dish at about 3 mm thickness and allowed to polymerize overnight at 60 °C. Cylinders with 1.4 cm diameter were punched out of the polymerized PDMS. Silk solutions were added on top of the cylinders and allowed to dry overnight under constant air flow. Water annealing (WA) the silk films was performed as previously described with slight adjustments to time and temperature [42]. The dried silk films were placed in a vacuum chamber with a 1 L basin of water inside at 23 °C. The vacuum chamber was sealed and a vacuum was pulled to −80 kPa. The vacuum line was shut to allow the water to evaporate and build the water pressure in the chamber. The amount of time in the WA chamber is specified in each experiment.

2.3. Silk-aniline conjugation

Tyrosine residues on silk fibroin were conjugated to 4-(2-aminoethyl)-aniline, as previously described [43]. Briefly, a 6% silk solution was dialyzed against borate buffer (100 mM borate, 150 mM NaCl, pH 9.0) for 1 day prior to reaction. To create the diazonium salt of the aniline 34 mg of 4-(2-aminoethyl)-aniline (2.5×10^{-4} mol) (Sigma Aldrich) was initially dissolved in 1.25 mL acetonitrile (0.2 M). Then 625 μl of the aniline solution was mixed with 625 μl 1.6 M p-toluenesulfonic acid. The analine/toluene sulfonic acid mixture was combined 1:1 with 625ul 0.8 M $NaNO_2$ on ice and stirred for 15 min to create the diazonium salt of the aniline. The diazonium-aniline solution (125 μl) was added to 2 mL silk solution in borate buffer and 375 μl dH_2O to bring the total volume to 2.5 mL. The reaction was allowed to proceed for 10 min. Reacted silk-aniline solution was dialyzed (Slide-A-Lyzer Dialysis Cassettes 3500K MWCO, Thermo Fisher, Rockford, IL) against deionized water